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Dissecting The Molecular Basis Of The Scaffold Protein Jade In Hbo1 Histone Acetyltransferase (hat) Activity

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Dissecting The Molecular Basis Of The Scaffold Protein Jade In Hbo1 Histone Acetyltransferase (hat) Activity

Abstract

HBO1 is a member of the human MYST family of acetyltransferases that is evolutionarily conserved from yeast to human. HBO1 functions in the context of a multi-protein histone acetyltransferase (HAT) complex containing JADE1/2/3, HBO1, ING4/5 and Eaf6 to regulate DNA replication, transcription, and other important cellular processes. HBO1 is shown to be responsible for the majority of H4 acetylation throughout the human genome. HBO1 functions in many important biological processes such as interaction with the origin recognition complex (ORC) and loading of the minichromosome maintenance protein complex (MCM), highlighting its importance in DNA replication. In addition, HBO1 is heavily co-localized to the transcription start site and H3K4me3 mark, which is one of the post translational modifications (PTMs) recognized by the PHD domains of another subunit in the HBO1 HAT complex containing HBO1, JADE, ING, Eaf. The activity of HBO1 is heavily dependent on the regulatory proteins within the HBO1 HAT complex.

We demonstrate that the scaffold protein JADE functions as a platform to bring other subunits together forming a stable HBO1 HAT complex in addition to recruiting the substrate. Importantly, JADE is the main protein in the HBO1 HAT complex contributing to modulation of the activity and substrate specificity. Based on the fact that JADE contributes strongly to the overall activity of the HBO1 HAT complex, we dissected the molecular role of JADE in HBO1 histone acetyltransferase activity. We demonstrate that JADE contains 2 domains at the N-terminal end of the protein that cooperate to activate HBO1 activity towards H3/H4 substrate. Specifically, the Histone Core Binding Domain (HCBBD) of JADE1 binds to the core of the histone and HBO1 and the Histone Binding Domain (HHBD) binds to both the HAT domain and the histone core, orienting the HAT domain towards the substrate. In addition, we demonstrated that JADE specifically binds to H3/H4 over H2A/H2B histones suggesting that the substrate recruitment is through specific interaction between JADE and H3/H4 rather than non-specific charge interaction. We demonstrate that HBO1 contains an N-terminal Histone Binding Domain (HBD) that makes additional contacts to H3/H4, independent of JADE interactions with histones. However, the additional HBO1 binding domain does not contribute to the overall HAT activity. Together, these studies highlight the importance JADE in HBO1 function.

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DISSECTING THE MOLECULAR BASIS OF THE SCAFFOLD PROTEIN JADE IN HBO1
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Dedicated to My Mom and Dad

Acknowledgements

Dear Ronen,

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Dear the current and previous members of the Marmorstein lab,

I give you guys much respect for putting up with me on a daily basis.

Dear all my friends and family,

You know who you are and how much you mean to me. I love you guys.

ABSTRACT

Joseph Han

Ronen Marmorstein

HBO1 is a member of the human MYST family of acetyltransferases that is evolutionarily conserved from yeast to human. HBO1 functions in the context of a multi-protein histone acetyltransferase (HAT) complex containing JADE1/2/3, HBO1, ING4/5 and Eaf6 to regulate DNA replication, transcription, and other important cellular processes. HBO1 is shown to be responsible for the majority of H4 acetylation throughout the human genome. HBO1 functions in many important biological processes such as interaction with the origin recognition complex (ORC) and loading of the minichromosome maintenance protein complex (MCM), highlighting its importance in DNA replication. In addition, HBO1 is heavily co-localized to the transcription start site and H3K4me3 mark, which is one of the post translational modifications (PTMs) recognized by the PHD domains of another subunit in the HBO1 HAT complex containing HBO1, JADE, ING, Eaf. The activity of HBO1 is heavily dependent on the regulatory proteins within the HBO1 HAT complex.

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Table of Contents

Table of Contents

Acknowledgements	iii
Abstract	v
Table of Contents	vii
List of Figures.....	x
1.1 Histone Acetylation	2
1.2 HBO1 Histone Acetyltransferase (HAT) Complex	6
1.3 Role of auxiliary subunits within histone acetyltransferase complexes	12
1.4 Dissertation objective.....	16
Chapter 2- Scaffold protein JADE is important for assembly and activity of the HBO1 HAT complex.....	17
2.1 Abstract	18
2.2 Introduction	19
2.3 Results	22
2.3.1 JADE functions as a scaffold protein to bring other subunits together to form different HBO1 HAT complexes.....	22
2.3.2 Comparison of the Kinetic parameters of the 2-protein and 4-protein HBO1 complexes indicates that JADE is sufficient for maximal HBO1 activity	25
2.3.3 The N-terminal Domain I of JADE forms a stable complex with the HBO1 HAT domain.	27
2.3.4 Domain I of JADE is sufficient for activation of HBO1 acetyltransferase activity towards H4 peptide	37
2.4 Discussion.....	41
2.5 Materials and Methods:	41
2.5.1 Full-length HBO1 HAT complexes.....	41
2.5.2 Suprose-6 size exclusion chromatography.	42
2.5.3 JADE1/2/3 N-terminal sequence alignment.....	42
2.5.4 Recombinant HBO1 and JADE1 protein production in bacteria.....	42
2.5.5 Histone acetyltransferase(HAT) activity assay.....	43
Chapter 3 -The scaffolding protein JADE1 physically links the acetyltransferase subunit HBO1 with its histone H3/H4 substrate.....	44

3.1 Abstract	45
3.2 Introduction	45
3.3 Results	47
3.3.1 <i>A 21-residue segment of JADE1 directly contacts the HBO1 HAT domain and histone substrate to facilitate acetylation of free histones</i>	49
3.3.2 <i>JADE1 contains a second histone binding domain that contributes to HBO1 acetylation of histone H4 substrate by anchoring the HBO1/JADE1 complex to the histone core</i>	53
3.3.3 <i>JADE1-HCBD shows selectively for free histone H3/H4 over H2A/H2B and nucleosomes</i>	57
3.3.4 <i>JADE1-HHBD and JADE1-HCBD bind independently to their respective partners, but cooperate to potentiate HBO1-HAT acetylation</i>	60
3.3.5 <i>HBO1 contains a region N-terminal to the HAT domain that binds H3/H4 independent of JADE1 binding to H3/H4</i>	62
3.3.6 <i>HBO1 may contain an auto-inhibitory domain</i>	65
3.3.7 <i>Cellular experiments with JADE1 deletions recapitulate the role of JADE1 in histone and HBO1 interactions and HBO1 acetyltransferase activity</i>	67
3.4 Discussion	69
3.5 Materials and Methods.....	73
3.5.1 <i>JADE1/2/3 N-terminal sequence alignment</i>	73
3.5.2 <i>Recombinant HBO1 and JADE1 protein production</i>	73
3.5.4 <i>Histone and nucleosome core particle production</i>	74
3.5.5 <i>Pull down assay (GST-/MBP-)</i>	74
3.5.6 <i>Histone acetyltransferase(HAT) activity assay</i>	75
3.5.7 <i>JADE1-HBO1 complexes cotransfection in 293T cells</i>	75
3.5.8 <i>Immunoblotting</i>	76
3.5.9 <i>HAT activity assays on purified JADE1 complexes</i>	76
Chapter 4 -Conclusion and Future direction.....	77
4.1 Overview	78
4.2 JADE as the scaffold protein for the HBO1 HAT complex.....	78
4.3 Kinetic approach to understanding the possible allosteric affects of JADE1 on HBO1 HAT domain.....	79
4.4 H3/H4 core chemical acetylation.....	80
4.5 Purification of the HBO-HAT holo complex.....	80

Appendix A -Biochemical studies on the Nat10 acetyltransferase	82
A.1 Abstract.....	83
A.2 Introduction.....	83
A.3 Result.....	85
A.4 Discussion	91
A.5 Materials and Methods	94
<i>A.5.1 NAT10 orthologs sequence alignment</i>	94
<i>A.5.2 Recombinant NAT10 protein production</i>	94
<i>A.5.3 Tubulin Purification</i>	94
<i>A.5.4 NAT10 Acetylation assays</i>	95

List of Figures

Figure1. 1 Table of different members of the human histone acetyltransferases (HATs).....	3
Figure1. 2 Acetylation and regulation of histone acetylation.....	5
Figure1. 5 Common features of the MYST histone acetyltransferase (HAT) complexes.....	10
Figure1. 6 Model for alternate MSYT acetyltransferase assembly leading to different substrate specificities.....	11
Figure1. 7 X-Ray crystallographic structures of histone acetyltransferase (HAT) complex.....	15
Figure 2. 1 The schematic representation of 2 different HBO1 HAT complex formation due to splice variants on JADE	21
Figure 2. 2 JADE functions as a scaffold protein to assemble different HBO1 HAT complex.....	24
Figure 2. 3 JADE is the main player of the HBO1 HAT complex in catalytic contribution.	26
Figure 2. 4 Primary sequence alignment of JADE1/2/3/ Paralog.	28
Figure 2. 5 Primary sequence alignment of HBO1 and its orthologs from various species all the way from yeast to human.....	30
Figure 2. 6 JADE domain I forms a complex with HBO1 HAT domain.	32
Figure 2. 7 JADE1 projects the N-terminal end of HBO1 HAT domain.	34
Figure 2. 8 HBO1 is hydrolyzed at the flexible region in the HAT domain.....	36
Figure 2. 9 HBO1 HAT domain responses to addition of JADE Domain I in acetyltransferase activity	38
Figure 2. 10 JADE1 Domain I (1-188) is able to stimulate activity of the isolated HBO1 HAT domain towards H4 peptides.....	40
Figure 3. 1 HBO1 acetyltransferase activity on different histone substrates.....	48
Figure 3. 2 An N-terminal region of JADE1 binds HBO1.....	51
Figure 3. 3 JADE1 requires an additional N-terminal region for H3/H4 binding and HBO1 HAT potentiation.	53
Figure 3. 4 The additional JADE1 N-terminal HCBP specifically binds the histone core region.....	56
Figure 3. 5 JADE1 selectively binds to H3/H4 over H2A/H2B and the nucleosome core particle.	59
Figure 3. 7 HBO1-FL contains an N-terminal H3/H4 binding domain that binds histones independently of JADE1.....	64
Figure 3. 8 Activity comparison of HBO1 full length vs HBO1 HAT domain.....	66
Figure 3. 9 JADE1 HHBD and HCBP are both required for HBO1 acetyltransferase activity on histones in cells.....	68
Figure 3. 10 Schematic of the HBO1, JADE1, H3/H4 complex based on the data obtained in this study.....	72
Figure A. 1 NAT10	86
Figure A. 2 Purification of NAT10 acetyltransferase domain.....	87

Figure A. 3 ITC of NAT10(494-753) with A-CoA, Acetonyl-CoA, CoA, Remodelin	89
Figure A. 5 Radioactive assay of NAT10 acetyltransferase activity.....	92
Figure A. 6Radioactive assay of NAT10 acetyltransferase activity.....	93

Chapter 1 - Histone tail modifications and the important players

Section 1.3 excerpt was taken from a review in preparation for submission to Protein Science.

1.1 Histone Acetylation

Eukaryotic nucleosomes, comprised of two copies each of H2A, H2B, H3, and H4, are assembled into an octomer, which is wrapped in ~150 basepairs of DNA. The chain of these repeating units of assembled nucleosomes are subjected to different post translational modifications (PMTs) that aid in conformational switching between euchromatin and heterochromatin leading to either gene expression or repression respectively (Kornberg, 1974). Histone post translational modifications (PTM) such as acetylation, phosphorylation, SUMOylation, ubiquitination, and methylation introduce new functional groups to different histone proteins at specific residues. Together, different histone modifications have been shown to work through a combinatorial effect (referred to as the histone codes (Strahl and Allis, 2000)) that function in various cellular signaling events to carry out DNA-templated biological processes in response to external or internal signals (Drazic and Winter, 2014; Verdin and Ott, 2015). More than 50 years ago, Allfrey and coworkers first discovered that histones are acetylated to mediate gene transcription (Allfrey and Mirsky, 1964), but it was not until about 30 years later that Allis and coworkers isolated the first histone acetyltransferase (HAT) enzyme (Brownell et al., 1996). Since the discovery of the first HAT, acetylation has been one of the most extensively studied PTMs, leading to the discovery of more than 20 HATs (Drazic et al., 2016) (**Figure1.1**).

	HAT/KAT	New name	Subcellular localization	Representative substrates
GNAT	HAT1	KAT1	Nucleus	H2A, H4
GNAT	GCN5 (GCN5L2)	KAT2A	Nucleus	H3, CEBPB
GNAT	PCAF	KAT2B	Nucleus	H3, H4, ACLY
p300/CBP	CBP	KAT3A	Nucleus/Cytoplasm	H2A, H2B, H3, H4, NCOA, FOXO1
p300/CBP	P300	KAT3B	Nucleus/Cytoplasm	H3, FOXO1, HDAC1, SIRT2, ALX1
	TAF1 (TAFII250)	KAT4	Nucleus	H3, H4
MYST	TIP60/PLIP	KAT5	Nucleus/Cytoplasm	H2A, H4, FOXP3
MYST	MOZ/MYST3	KAT6A	Nucleus	H3, H4, p53, RUNX2
MYST	MORF/MYST4	KAT6B	Nucleus	H3, RUNX2
MYST	HBO1/MYST2	KAT7	Nucleus	H4
MYST	MOF/MYST1	KAT8	Nucleus	H4, p53
GNAT	ELP3	KAT9	Nucleus/Cytoplasm	H3, H4, α -tubulin
	TFIIIC90 (GTF3C4)	KAT12	Nucleus	H3
	SRC-1 (NCOA1)	KAT13A	Nucleus	H3, H4
	SRC-3 (TRAM1, NCOA3, ACTR)	KAT13B	Nucleus/Cytoplasm	H3, H4
	SRC-2 (TIF2, GRIP1, bHLHe75, NCOA2, P160)	KAT13C	Nucleus	H3, H4
	CLOCK	KAT13D	Nucleus/Cytoplasm	ARNT1/BMAL1, NR3C1/GR
	ATF-2 (CREB2, CREBP1)		Nucleus/Cytoplasm	H2B, H4
GNAT	ATAT1		Cytoplasm/Membrane	α -tubulin
	ACAT1		Mitochondria	PDP
	NAT10		Nucleus	Histones, α -tubulin
	GCN5L1 (BLOC1S1)		Mitochondria	ATP5A1, NDUF9

Figure1. 1 Table of different members of the human histone acetyltransferases (HATs).

The list shows their names, localizations, and substrates

Drazic et al. (2016) *Biochim Biophys Acta*.

Histone acetylation, a covalent modification on lysine residues within histone tails H2A, H2B, H3 and H4, regulates local and global chromatin dynamics—thus playing an important role in modulating gene expression in all living eukaryotic organisms. Generally, hyperacetylation and hypoacetylation are observed in transcriptionally active and inactive regions of the chromatin, respectively (Kornberg and Lorch, 1999; Sapountzi and Cote, 2011; Strahl and Allis, 2000; Struhl, 1998; Thomas and Voss, 2007). This post-translational modification on the amino group of lysine side chains neutralizes the positive charge, which in turn leads to an alteration in structure of the amino acid as well as the relative local hydrophobicity (Drazic et al., 2016; Sapountzi and Cote, 2011). This affects transcription in two major ways. First, the altered physiochemical properties of the lysine residue on the histone tails are believed to directly participate in making chromatin more accessible for gene activation by loosening up the interaction with the neighboring DNA (**Figure1.2A**). Second, lysine acetylation generates docking recognition sites for various gene-modulating proteins containing bromodomains or other acetyl-lysine recognition domains (Drazic et al., 2016; Havasi et al., 2013; Jenuwein and Allis, 2001; Peterson and Laniel, 2004; Taverna et al., 2007; Turner, 2000) (**Figure1.2B,C**). Histone acetylation is reversible and is mediated by histone *de*acetylase (HDAC), whose activity is generally correlated with gene repression or silencing (Allis et al., 2007). Together, the tight regulation of acetylation and deacetylation on lysine residues within histones by HATs and HDACs is very important to maintain normal gene expression. Due to the importance of HATs and HDACs in regulating gene expression, the altered activity of these enzymes has been connected to various diseases such as cancer, neurodegenerative diseases and cardiovascular disorders (Haberland et al., 2009a; Haberland et al., 2009b; Saha and Pahan, 2006).

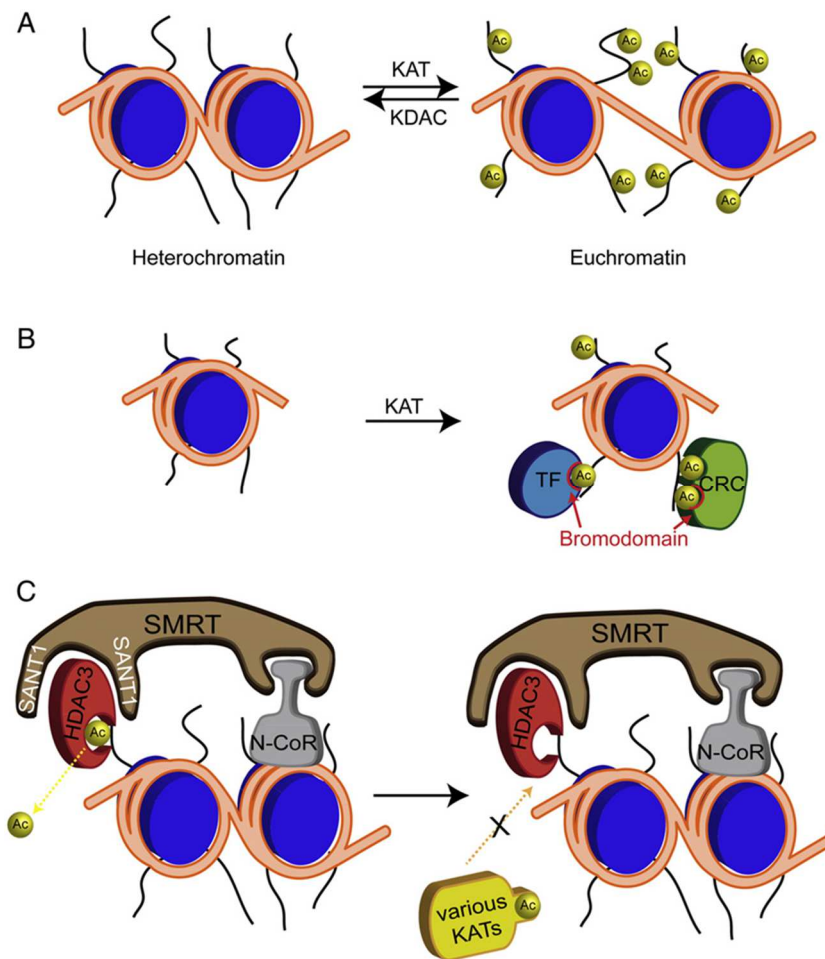


Figure1. 2 Acetylation and regulation of histoneacetylation.

(A) This reversible histoneacetylation leads to conformational changes from heterochromatin to the more open euchromatin structure. The reverse mechanism is done by Lysine deacetylases (KDAC). (B) The acetylated Lysine is recognized by the bromodomain within a larger protein complexes such as transcription factors (TFs) or chromatin remodeling complexes (CRCs) (C) Transcriptional repression is regulated by specific repressors, recruiting histone deacetylases (HDACs). Drazic et al. (2016) *Biochim Biophys Acta*.

The addition of acetyl groups on histone tails is catalyzed by HATs and are categorized into different families based on sequence conservation, called Gcn5/PCAF, MYST, p300/CBP and Rtt109 (Drazic et al., 2016) (**Figure1.1**). Each of the HAT families also contain structural

homology, conserved catalytic properties, and specificity toward their respective cognate substrate (Marmorstein and Zhou, 2014) (**Figure 1.3**). Most importantly, HATs function as members of multi-protein complexes where catalytic activation and substrate specificity can be modulated by other subunits within the complex (Berndsen and Denu, 2008; Marmorstein and Zhou, 2014).

1.2 HBO1 Histone Acetyltransferase (HAT) Complex

HBO1 (KAT7) is a member of the human MYST family of acetyltransferases that is evolutionarily conserved from yeast to human. The name MYST (Moz, YBF2, Sas2p, Tip) is derived from the founding members of this acetyltransferase family. The MYST family of acetyltransferases is characterized by a highly conserved MYST domain, and is further distinguished into three different subgroups depending on the domain compositions outside of the catalytic domain (Thomas and Voss, 2007) (**Figure 1.4**). Other members of the human MYST family include TIP60(KAT5), MOZ(KAT6A), MORF(KAT6B), and MOF(KAT8). HBO1 is responsible for the majority of H4 acetylation throughout the human genome, and directly regulating DNA replication through this activity (Doyon et al., 2006; Foy et al., 2008; Miotto and Struhl, 2010). The lethality of HBO1 knockout mice also suggests that HBO1 HAT activity is required in modulating expression of key genes in development and embryonic patterning (Kueh et al., 2011). Finally, the importance of HBO1 catalytic activity is highlighted by the findings that a HBO1 catalytic defective mutant inhibits interaction with ORC (Iizuka and Stillman, 1999) and loading of MCM complex onto the origin, indicating that the HBO1 HAT complex functions as a DNA replication co-activator in addition to its other roles (Havasi et al., 2013; Miotto and Struhl, 2008, 2010). Smith and coworkers have observed over-expression of HBO1 in MCF7 and Saos-2 established cancer cell lines and in a subset of human primary cancers such as carcinomas of testis, breast, and ovary (Iizuka et al., 2009).

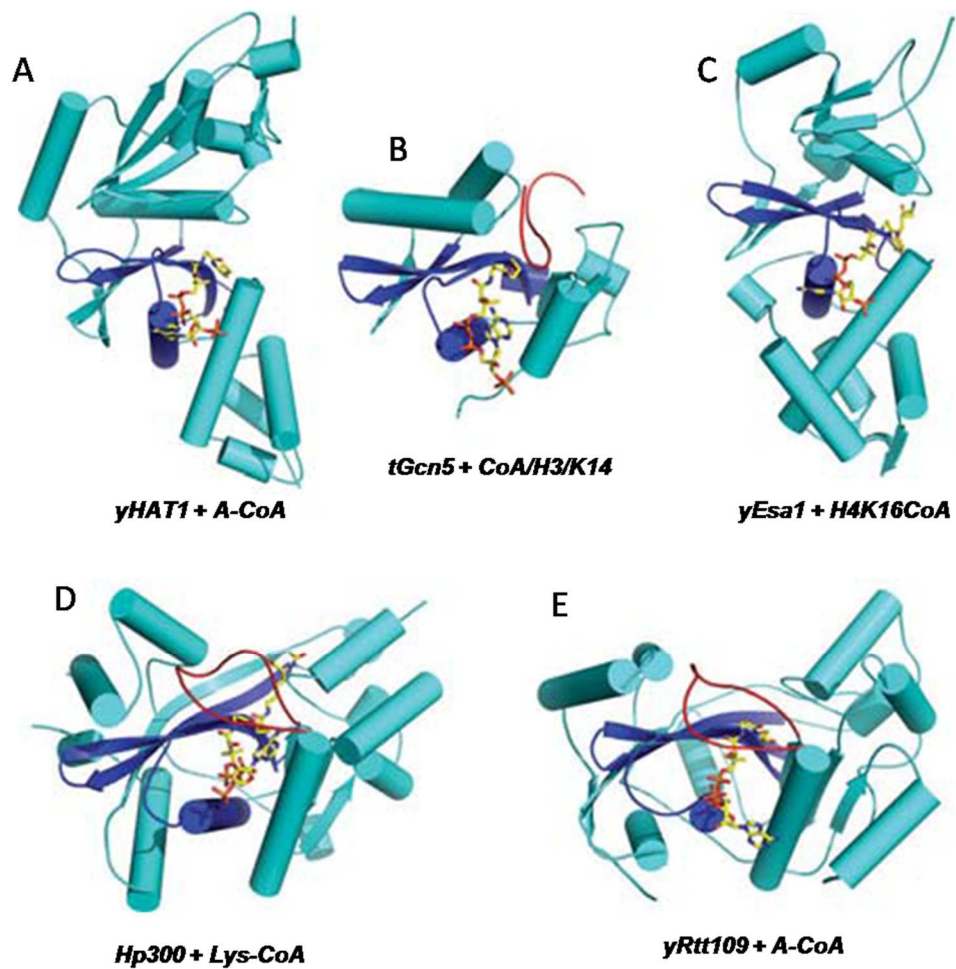


Figure1. 3 Over all structure of 5 HAT subfamilies illustrated as cartoons. (Blue) structurally conserved core region (aqua) flanking N- and C- termini Cofactor shown as stick figure.

(A)YeastHAT1/AcCoA (PDB:1BOB) (B)*Tetrahymena* Gcn5/CoA/histone H3 (PDB : 1PUA) with the histone H3 peptide shown in red (C)Yeast Esa1/H4K16-CoA (PDB : 3TO6) (D)*Human* p300/Lys-CoA (PDB : 3BIY) with the substrate-binding loop shown in red (E)Yeast Rtt109/CoA (PDB : 3D35) with the substrate-binding loop shown in red. Marmorstein et al. (2014) Cold Spring Harb Perspect Biol.

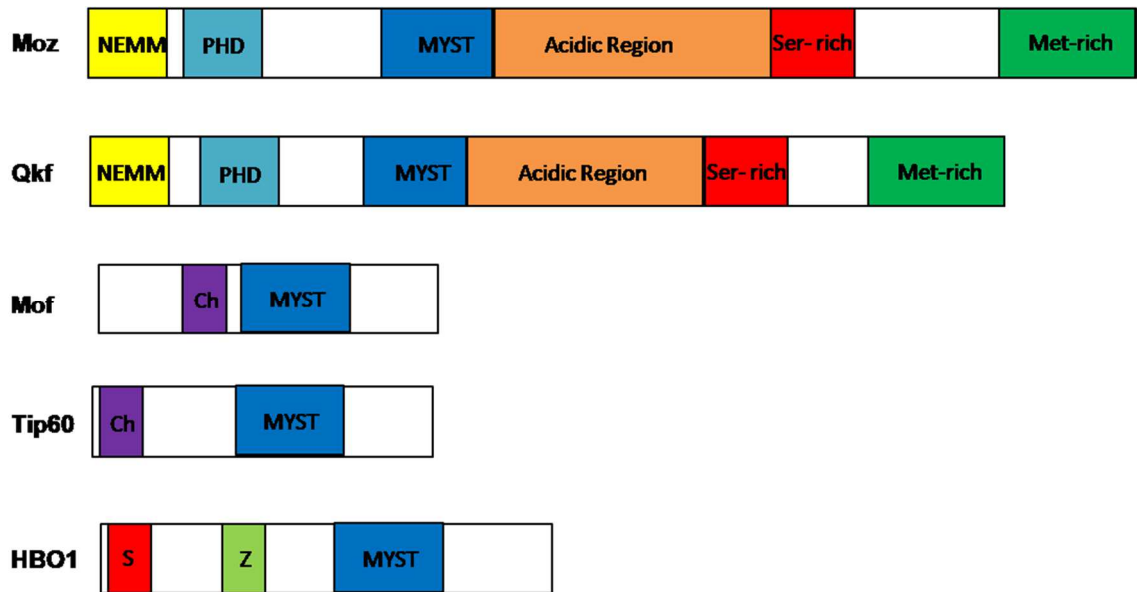


Figure1. 4 Schematic diagram comparing MYST family proteins and their protein domains.

Each domains are indicated in different colors

HBO1 functions in the context of two multi-protein histone acetyltransferase complexes containing ING4/5, MEAF6 and paralogs JADE1/2/3 for targeting the H4 tail or BRPF1/2/3 for H3K14 acetylation (Feng et al., 2016; Lalonde et al., 2013) (**Figure 1.5**). The activity and substrate selectivity of HBO1 is dependent on the proteins within the HBO1 HAT complex, which also have other functions in various cellular proliferation and tumor suppression pathways. For example, JADE1 was initially identified as a protein partner of the von Hippel-Lindau tumor suppressor to regulate cellular oxygen sensing (Zhou et al., 2002). A recent study demonstrated that an HBO1 complex containing the BRPF1 protein in place of JADE1 has specificity toward H3 tail acetylation, rather than the H4 tail acetylation preferred by the JADE1 containing complex (**Figure 1.6**). This suggests that JADE1 contributes specifically toward H4 tail acetylation by the HBO1 complex (Avvakumov et al., 2012; Havasi et al., 2013; Lalonde et al., 2013). Another member of the HBO1 complex, ING, is an inhibitor of growth protein responsible for growth regulation in eukaryotic organisms.

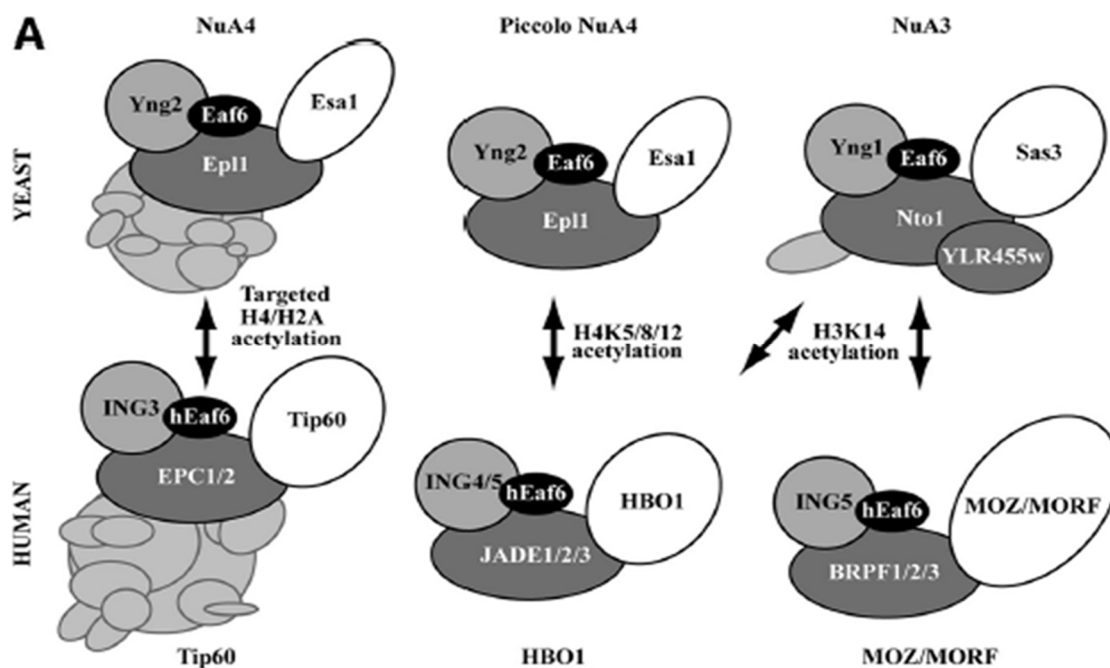


Figure1. 5 Common features of the MYST histone acetyltransferase (HAT) complexes.

Avvakumov *et al.* (2012) *Mol Cell Biol.*

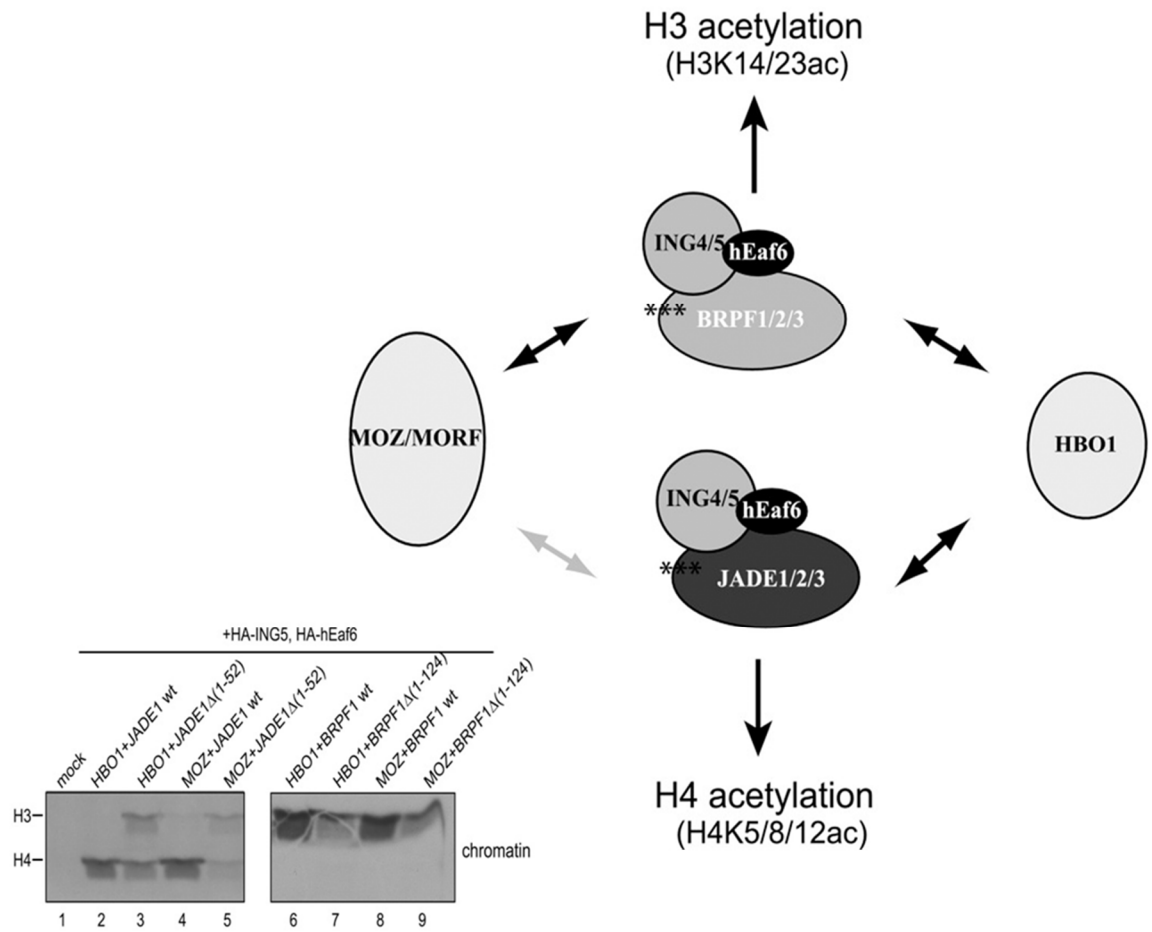


Figure1. 6Model for alternate MSYT acetyltransferase assembly leading to different substrate specificities.

Lalonde et al. (2013) *Genes & development*.

Mutation or down-regulation of ING genes leads to tumor developmental pathways(Piche and Li, 2010; Ythier et al., 2008). The HBO1 HAT complex contains three PHD fingers responsible for recognizing various methylated states of H3K4. JADE1 contains two of these motifs (termed PZP), which cooperate to enable acetylation of nucleosome particles and recognition of unmethylated H3K4(Avvakumov et al., 2012; Lalonde et al., 2013; Saksouk et al., 2009). ING4 contains the third PHD finger, which recognizes trimethylated H3K4 (H3K4me3). The H3K4me3 modification state has been shown to be correlated with the ability of the HBO1 HAT complex to acetylate H4(Doyon et al., 2006; Foy et al., 2008; Havasi et al., 2013; Lalonde et al., 2013; Saksouk et al., 2009). Through its recognition of H3K4me3, ING4 has been proposed to play a lynchpin role in the acetylation process. Thus ING4 can link its aforementioned tumor suppressor function with chromatin binding, transcriptional activity, and cellular proliferation by nucleating the complex recruitment process(Avvakumov et al., 2012; Piche and Li, 2010; Ythier et al., 2008). This is further substantiated by the activity of JADE1 spliced variants. Specifically, while the longer JADE1L form harbors tumor suppressor activity, the shorter JADE1 variant that lacks the ING4 binding domain but still binds to HBO1, called JADE1S, does not function as a tumor suppressor(Avvakumov et al., 2012). Together, JADE1 in the HBO1 HAT complex has been proposed to function as an intermediary to link HBO1 HAT catalytic activity to ING tumor suppressor activity through cross talk between two different epigenetic modifications of methylation and acetylation(Saksouk et al., 2009). The absence of any of the HBO1 HAT complex subunits leads to diminished acetylation activity on cognate substrates, implying that these HBO1 HAT complex subunits play important regulatory roles(Avvakumov et al., 2012; Lalonde et al., 2013; Saksouk et al., 2009).

1.3 Role of auxiliary subunits within histone acetyltransferase complexes

The NuA4 complex, containing catalytic subunit Esa1 as well as additional subunits Epl1, Yng2, and Eaf6, is the main player in global H4 acetylation in *S. cerevisiae*, where Esa1 serves as the only HAT essential for cell cycle (Smith et al., 1998). Recent crystallographic and low-

resolution EM analyses of the yeast NuA4 complex suggest that Esa1 recognizes its target residues on the histone tail through a mechanism of substrate specificity that depends on the other subunits of the complex. The tetrameric core of the NuA4 complex consists of Esa1, Epl1, Yng2, Eaf6, where the scaffold protein Epl1 serves as the platform for assembly of the complex (**Figure 1.7A**). Esa1 and the N-terminus of Epl1 associate to form the catalytic core, while Yng2, Eaf6 and the C-terminus of Epl1 form a helical bundle. The catalytic core and the helical bundle regions are connected by a loop in Epl1 referred to as the dual function loop (DFL), as it plays a key role in both active site rearrangement and in nucleosome binding. The 2.0 Å crystal structure of Esa1 in the absence of Epl1 suggests that the loop in between $\alpha 2$ - $\beta 7$ in the active site is highly conserved among MYSTs and is important for catalysis and for substrate binding (Yan et al., 2000) (**Figure 1.7B**). In the absence of Epl1, residues within the Esa1 $\alpha 2$ - $\beta 7$ loop interact with several $\alpha 2$ residues, but in the recent 3.5 Å crystal structure of the tetrameric NuA4 core complex, the $\alpha 2$ - $\beta 7$ loop residues R249, L264, and Y265 interact with a highly conserved FRR (F312, R313, R314) motif in Epl1 to reshape its substrate binding surface (Xu et al., 2016). This new binding surface has a preference for a substrate with a small residue (G or A) at the P-1 position, allowing Esa1 to specifically target H4 (K5, K8, K12, K16). Additionally, low-resolution cryo-EM studies and biochemical assays reveal that substrate specificity of the NuA4 complex is further achieved through the N-terminal portion of the Epl1 DFL, which associates with the nucleosome and helps position the NuA4 complex on the dish face of the nucleosome with the active site proximal to the H4 tail. (**Figure 1.7A**)

A recent 2.4 Å crystal structure of the HBO1 HAT domain in complex with an N-terminal fragment of BRPF2 reveals that BRPF2 binding modulates HBO1 substrate specificity through a mechanism differing from the Epl1/Esa1 interaction. The structure reveals that BRPF2 wraps around the C-terminal part of the HBO1 HAT domain with the interaction stabilized by a network of hydrogen bonds and Van der Waals interactions between the two proteins (**Figure 1.7C**). Unlike Esa1 in the NuA4 complex, the active site of HBO1 does not undergo any conformational

change when in complex with BRPF2. Further biochemical analysis highlights two different acidic patches within BRPF2 consisting of residue E41/E43/E45 and E62/D63/D64 that are responsible for binding and positioning the H3/H4 substrate proximal to the HBO1 active site (Tao et al., 2017). The crystal structure of HBO1-BRPF2 complex indicates that the histone binding site of BRPF2 is ~40 Å away from the active site, suggesting that BRPF2 may bind to the core of the H3/H4 histone, which positions the H3 tail in close proximity to the active site of HBO1 (Tao et al., 2017).

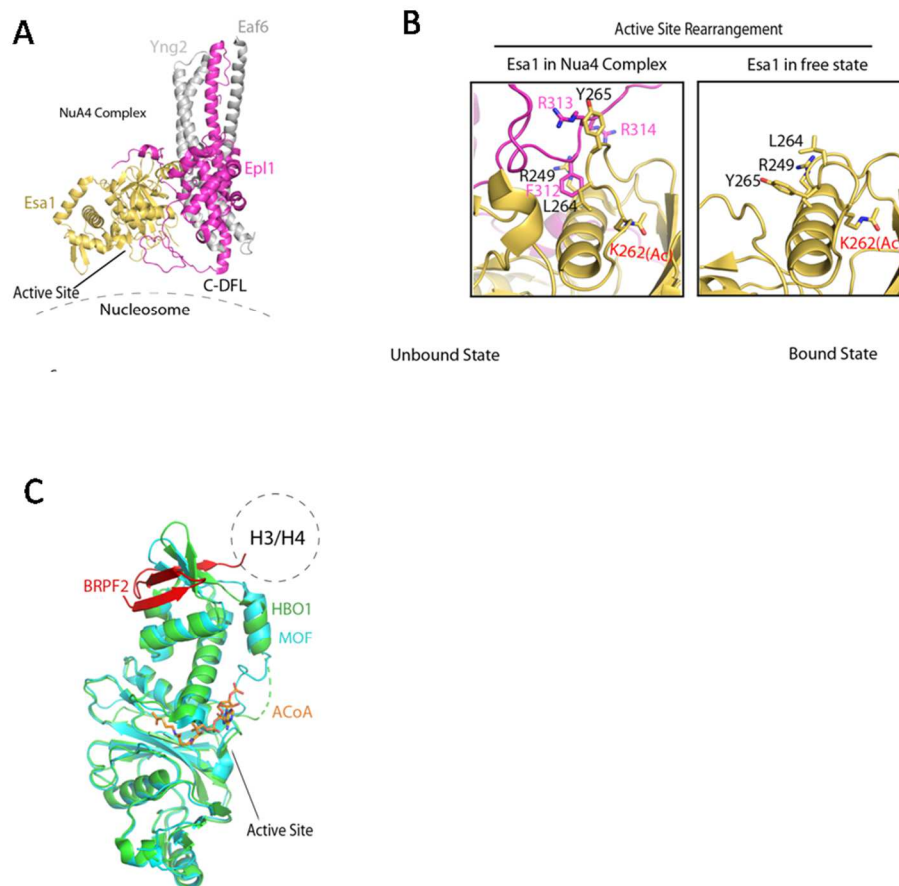


Figure1. 7X-Ray crystallographic structures of histone acetyltransferase (HAT) complex.

(A) 2.0 Å crystal structure of the *S. cerevisiae* NuA4 complex containing Esa1, Epl1, Yng2 Eaf6. The active site of Esa1, DFL of Epl1 and the position of the NuA4 complex relative to nucleosome are indicated. (PDB: 5J9U) (B) Active site comparison of Esa1 in the NuA4 complex versus the free state. (PDB: 3TO9) (C) 2.4 Å crystal structure of the human HBO1 HAT domain in complex with an N-terminal fragment of BRPF2 (PDB: 5GK9) overlaid with MOF (PDB: 2GIV) bound to A-CoA with substrate binding site indicated.

1.4 Dissertation objective

Since the discovery of the MYST family, biochemical and structural analysis has led to a detailed understanding of the catalytic domain of these proteins. However, how the subunits of the respective MYST HAT complex contribute to the activity and substrate selectivity of the complex was not known. For my dissertation, I dissected the molecular role of the subunits of the HBO1 complex for HBO1 acetyltransferase activity and substrate specificity. Together, my studies have important implications for understanding the molecular basis for HBO1 regulation within the catalytic complex and point to the particular importance of the scaffold protein JADE for function of the HBO1 complex. My studies also have broader implications for the development of HBO1-selective MYT inhibitors.

Chapter 2- Scaffold protein JADE is important for assembly and activity of the HBO1 HAT complex

2.1 Abstract

HBO1 HAT complex has been shown to be responsible for global H4 acetylation. Previous studies have demonstrated that JADE serves as a scaffold protein to assemble HBO1 HAT complex. Through a DNA splice event, JADE can assemble 2 different biologically relevant forms of HBO1 HAT complexes. Subunits within the HBO1 HAT complex possess chromatin recognition domain that allow the complex to be properly localized throughout chromatin. Although we have some information about how the complex may be recruited to chromatin, the relative contributions of subunits of the HBO1 HAT complex to the catalytic activity of HBO1 has not been previously dissected. In this chapter, we demonstrate that JADE is the main subunit in the HBO1 HAT complex that contributes to assembly and the overall activity of the HAT complex towards H4 substrate.

2.2 Introduction

The human MYST family of HATs, comprised of MOZ, Tip60, HBO1, MORF, and MOF, are evolutionarily conserved with *S. cerevisiae* Esa1 and characterized by a highly conserved MYST fold within the catalytic domain (Marmorstein and Zhou, 2014). The structure of the Gcn5 and PCAF catalytic HAT domain bound to an H3K14-containing peptide reveals extensive backbone interaction with surrounding residues on the H3 tail, thus demonstrating that the catalytic Gcn5/PCAF HAT domain can bind its lysine substrate independently (Rojas et al., 1999). In contrast to the Gcn5/PCAF HATs, MYST family HATs are thought to have lower affinity towards their substrates and are generally more promiscuous toward multiple lysine residues within the same histone tails, thus requiring other subunits of their respective complexes to provide substrate specificity (Marmorstein and Zhou, 2014; Rojas et al., 1999).

HBO1, in particular, functions in the context of a multi-protein HAT complex containing JADE1/2/3, HBO1, ING4/5 and Eaf6 to regulate DNA replication, transcription, and other important cellular processes (Avvakumov et al., 2012) (**Figure 2.1**). The activity of HBO1 is heavily dependent on the regulatory proteins within the HBO1 HAT complex, which otherwise have their own functions in cellular proliferation and tumor suppression pathways. For example, JADE1 was initially identified as a protein partner of von Hippel-Lindau tumor suppressor to regulate cellular oxygen sensing (Zhou et al., 2002). A recent study demonstrated an HBO1 complex containing the BRPF1 protein in place of JADE1 has specificity for H3 acetylation, rather than the H4 acetylation preferred by the JADE1 containing complex. This suggests JADE1 plays an important role for specifying H4 acetylation by the HBO1 complex (Lalonde et al., 2013). Another member of the complex, ING, is an inhibitor of growth protein responsible for growth regulation in eukaryotic organisms. Mutation or down-regulation of ING genes leads to tumor developmental pathways (Piche and Li, 2010; Ythier et al., 2008). The absence of any of the HAT complex subunits leads to diminished acetylation activity on the cognate substrates, implying that these HBO1 HAT complex subunits play important regulatory roles (Avvakumov et al., 2012;

Lalonde et al., 2013; Saksouk et al., 2009). Lastly, Eaf6, is a MYST associated protein with an unknown function.

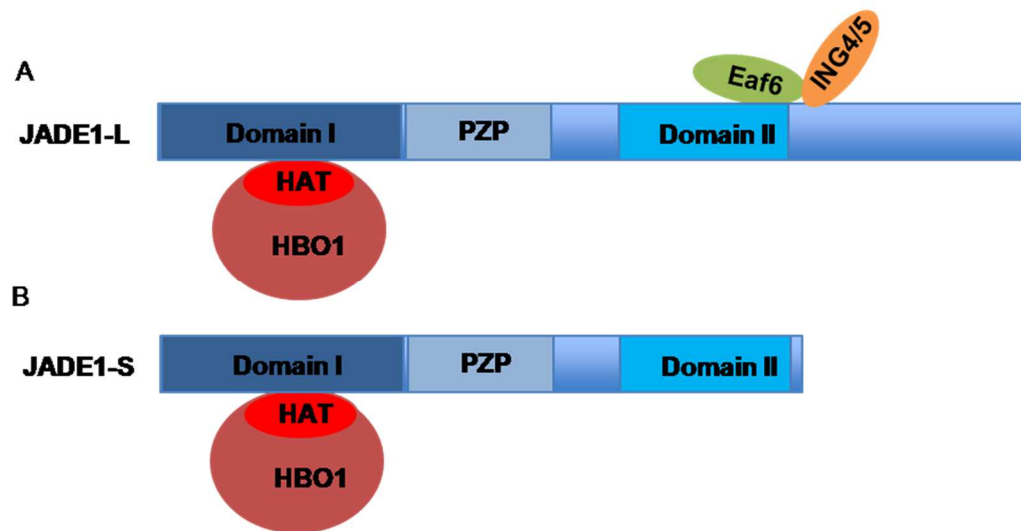


Figure 2. 1The schematic representation of 2 different HBO1 HAT complex formation due to splice variants on JADE

(A) JADE1-L forms a tetrameric HBO1 HAT complex with HBO1,Eaf6,ING4/5. (B) JADE1-S forms a dimeric HBO1 HAT complex with HBO1.

2.3 Results

2.3.1 JADE functions as a scaffold protein to bring other subunits together to form different HBO1 HAT complexes

Previous studies of the HBO1 HAT complex suggest that other subunits of the complex are important for HBO1's activity. However, the actual mechanism of how these subunits modulate the activity of the catalytic enzyme in the HAT complex is still unknown. As mentioned earlier, the HBO1 HAT complex exists as two different forms with different functions in the cell. The formation of these two different HBO1 HAT complexes is due to a DNA splicing event that occurs on JADE leading to either JADE short (JADE-S) or JADE long (JADE-L) variants. The main difference in between the two variants is that JADE-S forms a heterodimeric complex with HBO1 whereas JADE-L forms a heterotetrameric complex with HBO1/ING/Eaf (Figure 2.1). To study the catalytic contribution of each subunit in the HBO1 HAT complex, we initially set out to study if two different forms of the HBO1 HAT complex could be assembled and purified from recombinant proteins. Baculovirus infected Sf9 cells were used to express full-length HBO1 assembled as either 4 protein HBO1 HAT complex (HBO1/JADE3/ING4/Eaf6), or 2 protein HBO1 HAT complex (HBO1/JADE3). The cells were co-infected with expression plasmids expressing each protein for the 2 and 4 protein complexes. HBO1 complexes were purified to homogeneity using affinity and gel filtration chromatography. Specifically, the soluble fraction of the cell lysate was incubated with antibody based FLAG resin followed by 3XFLAG peptide elution. For the cells that were co-infected with 4 protein expression vectors, Flag-HBO1 co-purified with HA-JADE3, HA-ING4, HA-Eaf6 in solution and the complex remained stably associated. The co-purification of the full HBO1 HAT complex was further verified by running the protein on a Superose-6 size exclusion column (Figure 2.2 in blue). The protein complex co-migrated on the Superose-6 column eluting at a molecular marker that was consistent with a 1:1:1:1 globular protein complex, informing us that the proteins were likely folded correctly. In a similar fashion, the 2-protein complex containing Flag-HBO1 and HA-JADE3 was prepared and also co-migrated on the

Superose-6 column consistent with a 1:1 globular protein complex verifying their proper fold (**Figure 2.2 in red**). After the 4-protein and 2-protein HBO1 HAT complex was purified, the presence of JADE3, ING4, Eaf6 or JADE3 was confirmed by anti-HA western blot, respectively (data not shown).

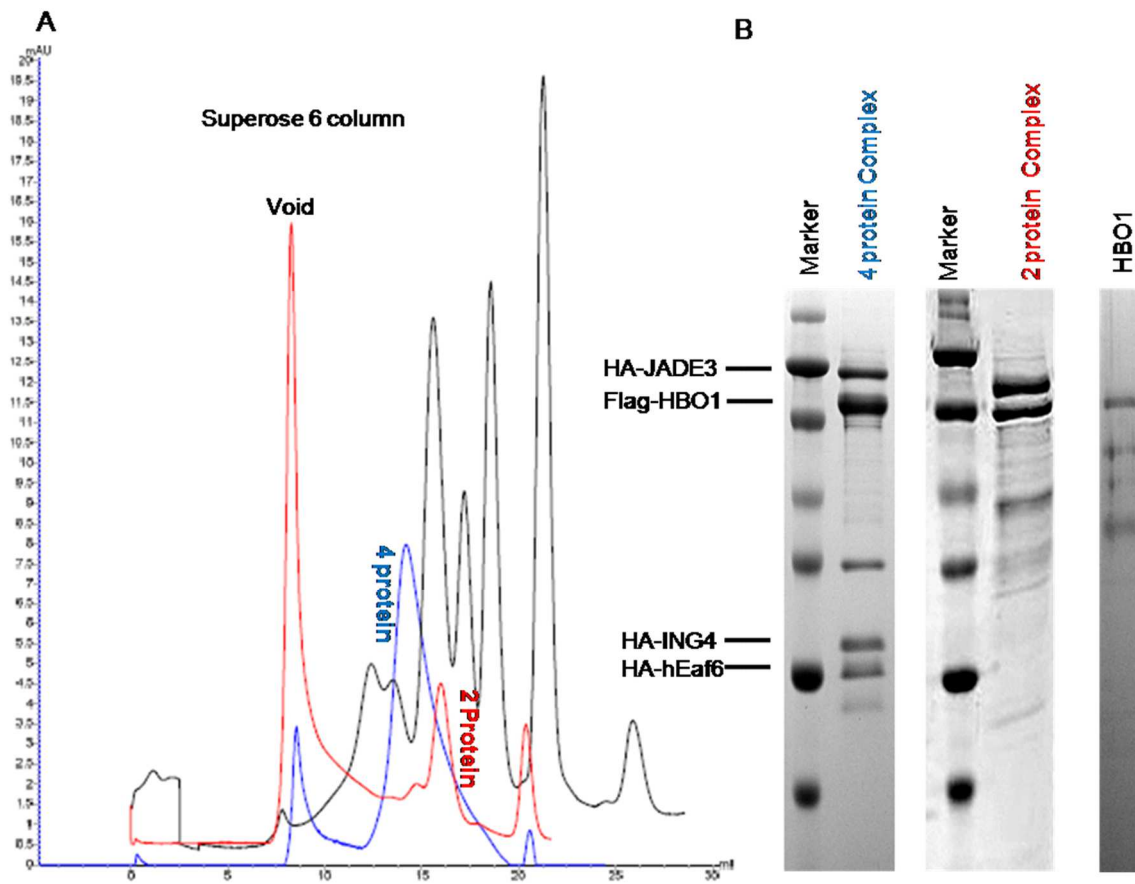


Figure 2. 2JADE functions as a scaffold protein to assemble different HBO1 HAT complex

(A) Red – 2 protein complex (Flag-HBO1/HA-JADE3) ~140KDa, Blue – 4 protein (Flag-HBO1/HA-JADE3/HA-ING4/ HA-Eaf6) ~200KDa, Black – Standard (670KDa, 158KDa, 44KDa, 17KDa, 1.35KDa) B. SDS-PAGE gel of the 2 protein complex and 4 protein complex post Suprose 6 gel filtration chromatography and HBO1 after Flag affinity purification.

2.3.2 Comparison of the Kinetic parameters of the 2-protein and 4-protein HBO1 complexes indicates that JADE is sufficient for maximal HBO1 activity

As described earlier, the HBO1 HAT complex exists as 2 biologically relevant forms either containing HBO1/JADE/ING/Eaf or HBO1/JADE. These 4-protein or 2-protein HBO1 HAT complexes have different functions in cells (Avvakumov et al., 2012) (**Figure 2.1**). To elucidate the kinetic contribution of the ING4 and Eaf6 subunits of the 4-protein HBO1 complex, we compared the steady state parameters, V_{\max} and K_m , of the 2- and 4-protein HBO1 complexes using substrate saturation curves with A-CoA (~500uM) and H4 peptide (~2mM). We employed a radioactive acetylation filter-binding assay described in the materials and method section. Using this method, we used the Michaelis-Menten kinetic model to obtain the steady state parameters. Although the curves did not fit perfectly with the Michaelis-Menten model, we were able to calculate the K_m and V_{\max} values of the 2 and 4 protein HAT complexes (**Figure 2.3**). Using the Michaelis-Menten curve, we demonstrated that the K_m and V_{\max} of these two HAT complexes were comparable to each other. Specifically, calculated K_m values of A-CoA were 3.1uM and 1.8uM for 4-protein and 2-protein complexes respectively. In addition, calculated K_m values of the H4 peptide was 229.1uM and 249.1uM for 4-protein and 2-protein complexes respectively. Furthermore, the V_{\max} values of A-CoA and H4 peptide were very similar for both complexes. In addition, HBO1 alone showed a much lower activity, which did not saturate under the tested conditions.

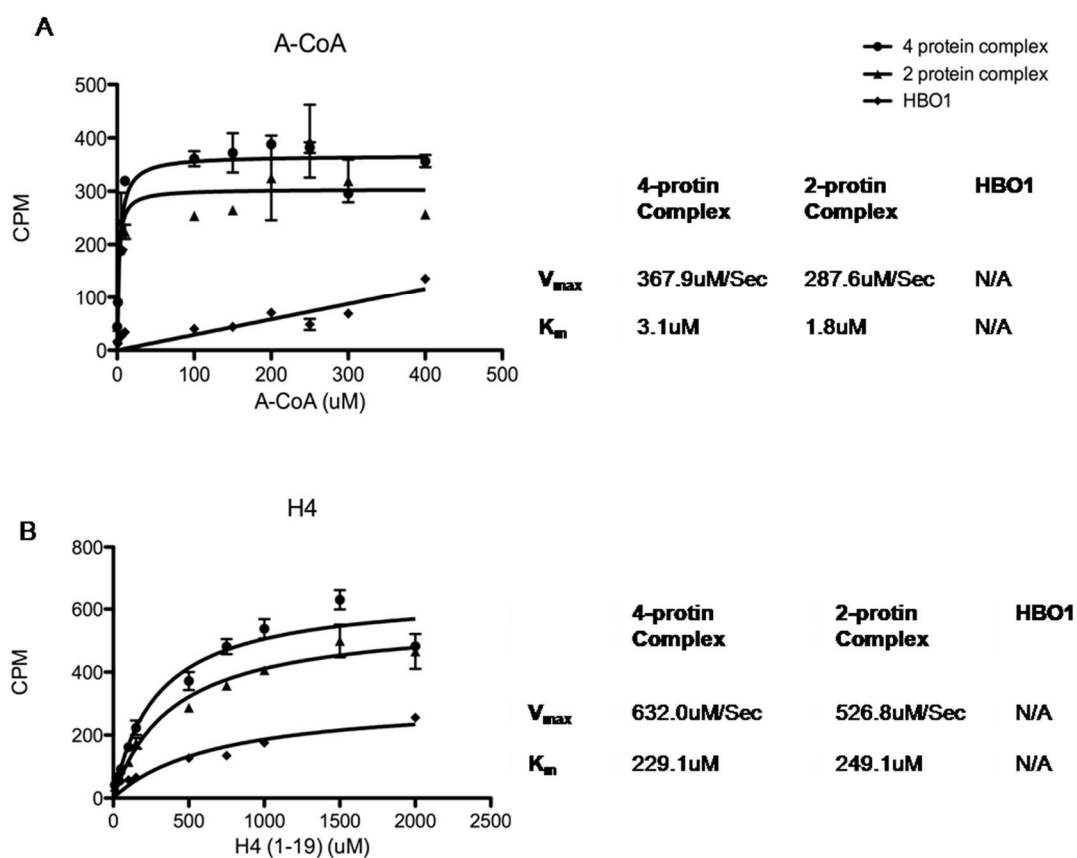


Figure 2. 3 JADE is the main player of the HBO1 HAT complex in catalytic contribution.

Saturation curve of A-CoA and H4 of the 2 protein complex (Flag-HBO1/HA-JADE3) and 4 protein complex (Flag-HBO1/HA-JADE3/HA-ING4/HA-Eaf6) and HBO1 using the Michaelis-Menten kinetic model. The output of the assay is displayed as counts per minute (CPM) (A) A-CoA (B) H4 peptide(1-19)

Although the HBO1 alone sample did not show much acetyltransferase activity towards H4 substrate, the kinetic parameters of the 4-protein and 2-protein HBO1 HAT complexes were relatively comparable to each other. The fact that the 2-protein complex was as active as the 4-protein complex indicates that ING4 and Eaf6 are possibly involved in non-catalytic functions of the HBO1 HAT complex, such as stability, or localization of the HAT complex to the correct region of the chromatin. Together, our data suggests that JADE is sufficient for HBO1 to reach its apparent maximum catalytic potential towards the substrate.

2.3.3 The N-terminal Domain I of JADE forms a stable complex with the HBO1 HAT domain.

After we determined that JADE is the main contributor of the HBO1 complex in enhancing HBO1 activity towards its substrate, we sought to further understand the interaction between these two proteins using the minimal interacting regions. The three different JADE paralogs that can interchangeably assemble within the HBO1 HAT complex are highly conserved (Avvakumov et al., 2012) (**Figure 2.4**). For our studies, we employed JADE1. Previously, Cote and coworkers identified Domain I of JADE1/2/3 to be responsible for interacting with the HAT domain of HBO1 (Avvakumov et al., 2012). Based on this, we sub-cloned an N-terminal DNA construct encoding JADE1 (1-188) containing Domain I into a bacterial expression vector. Using the sequence conservation of HBO1 orthologs from different species, we selected residues 302-611 as the HBO1 HAT domain, and sub-cloned a DNA fragment encoding this region to produce a recombinant HBO1 HAT domain in bacteria (**Figure 2.5**). These recombinant proteins were purified through a series of affinity, ion exchange, and size exclusion chromatography steps.

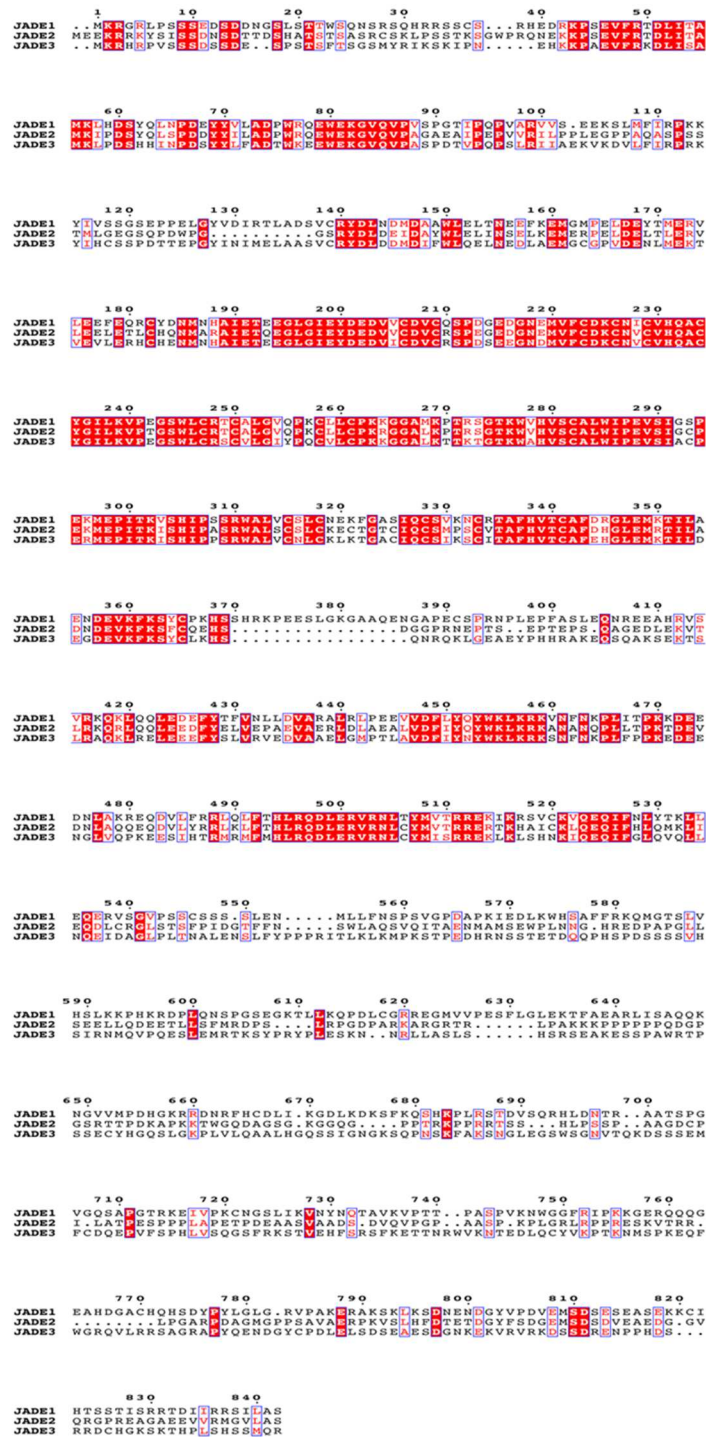


Figure 2. 4 Primary sequence alignment of JADE1/2/3/ Paralogs.

The red region indicates evolutionary conservation

ESA
 ESA
 elegans 146 MELDSDDDDPVNELDNLPIVIDDP...SYVLTKEHKEIFEQ...VKKSVSDRNEFSP
 Drosophila 496 ...V...PDYDLQLFREA...QAQASERIEDEL
 Xenopus 226 ...T...SEYDLELFRA...QARASDDLEKLR
 Mus 272 ...T...SEYDLELFRA...QARASDDLEKLR
 Human 302 ...T...SEYDLELFRA...QARASDDLEKLR
 Rattus 146 ...T...SEYDLELFRA...QARASDDLEKLR
 pombe 59 ...L...NQDDVRLFEKA...KTVADRTKDVYSFSDPIL...
 cerevisiae 108 ...S...LQDGVDSRENTDVMDLNLDNLVQGIKDENISHDEKCLR

ESA
 ESA 1 ...EVARVRN...LNR...IM...GK...E...E...P...W...F...S...P...I...E...L...D...E...F...I...Y...I...D...F...T...I...Q...Y...F...G...S...K...K...
 elegans 196 ...SEIYRSSKGEQARLP...ER...H...F...C...A...F...I...M...K...T...W...Y...G...S...P...P...A...E...F...I...N...V...K...K...L...F...I...C...E...F...C...F...Y...A...R...S...D...E...
 Drosophila 520 ...KDLPV...G...G...K...G...I...K...Y...I...S...M...G...K...Y...K...M...K...V...Y...O...S...P...P...D...A...A...R...L...P...K...M...Y...I...C...E...F...C...I...Y...Q...M...S...E...T...
 Xenopus 250 ...LGGQIT...EGSNM...K...T...I...F...G...Y...E...L...D...T...W...H...S...P...Y...P...E...E...Y...A...R...L...G...R...L...Y...M...C...E...F...C...I...Y...M...K...S...Q...T...
 Mus 296 ...LGGQIT...EGSNM...K...T...I...A...F...G...Y...E...L...D...T...W...H...S...P...Y...P...E...E...Y...A...R...L...G...R...L...Y...M...C...E...F...C...I...Y...M...K...S...Q...T...
 Human 326 ...LGGQIT...EGSNM...K...T...I...A...F...G...Y...E...L...D...T...W...H...S...P...Y...P...E...E...Y...A...R...L...G...R...L...Y...M...C...E...F...C...I...Y...M...K...S...Q...T...
 Rattus 170 ...LGGQIT...EGSNM...K...T...I...A...F...G...Y...E...L...D...T...W...H...S...P...Y...P...E...E...Y...A...R...L...G...R...L...Y...M...C...E...F...C...I...Y...M...K...S...Q...T...
 pombe 90 ...STQLRT...PPFQPTS...I...R...Y...L...Y...F...G...T...Y...R...I...K...P...W...T...S...P...Y...P...E...E...Y...S...C...A...K...N...L...Y...I...Q...E...S...C...I...Y...M...N...S...D...H...
 cerevisiae 150 ...TGGSTQNFHEVARVRN...LNR...IM...GK...E...E...P...W...F...S...P...I...E...L...D...E...F...I...Y...I...D...F...T...I...Q...Y...F...G...S...K...K...
 β1 β2 β3 TTT β4 QQ

ESA
 ESA 51 QVERYRK...K...C...T...L...R...H...P...P...G...N...E...I...Y...R...D...D...Y...V...S...F...F...E...I...D...G...R...K...O...R...T...N...C...R...N...L...C...L...L...S...K...L...F...L...D...R...K...T...L...Y...D...V...D...
 elegans 256 ...IMONHAK...K...C...M...L...R...A...P...P...G...E...I...Y...R...K...G...D...I...S...V...F...E...V...D...G...R...L...O...K...E...N...C...O...T...L...C...L...V...S...R...M...F...L...E...S...T...V...Y...D...T...E...
 Drosophila 571 ...GKRRHAE...K...C...V...W...R...H...P...P...G...E...I...Y...R...K...G...L...Q...V...Q...V...D...G...K...R...Y...K...O...Q...H...L...C...L...A...K...F...L...D...R...K...T...L...Y...D...V...E...
 Xenopus 304 ...ILRRHMA...K...C...V...W...K...H...P...P...G...E...I...Y...R...K...G...S...I...S...V...F...E...V...D...G...K...K...N...K...I...Q...N...L...C...L...A...K...F...L...D...R...K...T...L...Y...D...V...E...
 Mus 350 ...ILRRHMA...K...C...V...W...K...H...P...P...G...E...I...Y...R...K...G...S...I...S...V...F...E...V...D...G...K...K...N...K...I...Q...N...L...C...L...A...K...F...L...D...R...K...T...L...Y...D...V...E...
 Human 380 ...ILRRHMA...K...C...V...W...K...H...P...P...G...E...I...Y...R...K...G...S...I...S...V...F...E...V...D...G...K...K...N...K...I...Q...N...L...C...L...A...K...F...L...D...R...K...T...L...Y...D...V...E...
 Rattus 224 ...ILRRHMA...K...C...V...W...K...H...P...P...G...E...I...Y...R...K...G...S...I...S...V...F...E...V...D...G...K...K...N...K...I...Q...N...L...C...L...A...K...F...L...D...R...K...T...L...Y...D...V...E...
 pombe 146 ...VLQRHMK...K...C...S...W...S...Y...P...P...G...E...I...Y...R...D...K...N...I...S...I...F...E...V...D...G...Q...R...O...P...I...Q...N...L...C...L...A...K...M...F...L...H...S...M...L...Y...D...V...E...
 cerevisiae 210 ...QVERYRK...K...C...T...L...R...H...P...P...G...N...E...I...Y...R...D...D...Y...V...S...F...F...E...I...D...G...R...K...O...R...T...N...C...R...N...L...C...L...L...S...K...L...F...L...D...R...K...T...L...Y...D...V...D...
 α1 β5 β6 α2 TTT T

ESA
 ESA 111 PFFFYCMTRR...DELGHHLVGYFSKERE...ADGYNVACITLTPQYQRRMGYGLLIEFSY
 elegans 316 PFFFYLVITINDDIGCHFAGYFSKERE...PDVNNLSCITMTTPVQENGLGRFLIDISY
 Drosophila 631 PFFFYIMTLADVDGCHIVGYFSKHIPFLQEKNSFYNVSCITLTPVQRRMGYGLLIDISY
 Xenopus 364 PFFFYVMTEDNTGCHLIGYFSKEKNS...FLNVNVSCITLTPVQRRMGYGLLIDISY
 Mus 410 PFFFYVMTEDNTGCHLIGYFSKEKNS...FLNVNVSCITLTPVQRRMGYGLLIDISY
 Human 440 PFFFYVMTEDNTGCHLIGYFSKEKNS...FLNVNVSCITLTPVQRRMGYGLLIDISY
 Rattus 284 PFFFYVMTEDNTGCHLIGYFSKEKNS...FLNVNVSCITLTPVQRRMGYGLLIDISY
 pombe 206 PFFFYVLTETFDGQECKVIGYFSKERE...ASDYNVSCITLTPVQRRMGYGLLIEFSY
 cerevisiae 270 PFFFYCMTRR...DELGHHLVGYFSKERE...ADGYNVACITLTPQYQRRMGYGLLIEFSY
 β7 β8 TT β9 β10 η1 α3

ESA
 ESA 167 ELSSKRNKVGSPERPLSDGLLSYRAYWSDTLITLVEH...QKEITIDISSMTSM
 elegans 372 ALSRRKRWEGGPEQPLSELGRKAYGGYWRITIASCLGRKDELEFGSGISIKMIADDTGV
 Drosophila 691 ILTRVGGKIGSPERPLSDGLISYRSYWKVLLDYLCNR...SGNTAIKIDVSGETAIV
 Xenopus 420 LLSKVDEKVGSPERPLSDGLISYRSYWKVLLRLYLHNF...QGKEISIKIEISCEAV
 Mus 466 LLSKVDEKVGSPERPLSDGLISYRSYWKVLLRLYLHNF...QGKEISIKIEISCEAV
 Human 496 LLSKVDEKVGSPERPLSDGLISYRSYWKVLLRLYLHNF...QGKEISIKIEISCEAV
 Rattus 340 LLSKVDEKVGSPERPLSDGLISYRSYWKVLLRLYLHNF...QGKEISIKIEISCEAV
 pombe 262 LITQVGGKIGSPERPLSDGLVTVRSYWKMRVAKALLEI...TTPSINAIKSTSM
 cerevisiae 326 ELSSKRNKVGSPERPLSDGLLSYRAYWSDTLITLVEH...QKEITIDISSMTSM
 α4 β11 β12 α5

ESA
 ESA 221 TTTDILHTAKT...NLIRYYKQHII...F...L...NE...DILDRYNRLLAKK...RR...TIDPNRI
 elegans 432 NCHDILEVVCSSWAKPVDPDEKNHYK...EWDVDMVSIILRESEASTKTKVYDPECD
 Drosophila 746 YSYDIVSTLQALQMLKYWKGLHLV...LK...K...QDVLDEYERVKR...RGTFFKIDDCCLR
 Xenopus 475 NPVDIVSTLQALQMLKYWKGLHLV...LK...K...RODLIDEWASKDAKRRTNSNK...TMDPCLK
 Mus 521 NPVDIVSTLQALQMLKYWKGLHLV...LK...K...RODLIDEWIAKEAKRSNSNK...TMDPCLK
 Human 551 NPVDIVSTLQALQMLKYWKGLHLV...LK...K...RODLIDEWIAKEAKRSNSNK...TMDPCLK
 Rattus 395 NPVDIVSTLQALQMLKYWKGLHLV...LK...K...RODLIDEWIAKEAKRSNSNK...TMDPCLK
 pombe 316 VCDIVSTLLESLSVFYKVDPLKKYVLOLK...RD...ELENVYKAWNKKH...PQVNVNPKLLR
 cerevisiae 380 TTTDILHTAKT...NLIRYYKQHII...F...L...NE...DILDRYNRLLAKK...RR...TIDPNRI
 α6 β13 β14 α7 η2

ESA
 ESA 272 WKPPV...
 elegans 492 WPPRRKMRPSMDGYHELSKEEIEQDEQRRKSIQKTPVHVSMKATPTSTISLPVGSYKKVVR
 Drosophila 799 WPPFINIQSSSS...
 Xenopus 529 WPPPKGT...
 Mus 575 WPPPKGT...
 Human 605 WPPPKGT...
 Rattus 449 WPPPKGT...
 pombe 370 WPPYLGEQISNLLKENILIP...QKRLLDNSHHLD...SV...
 cerevisiae 431 WPPPVFTASQLRFAW...
 β15

Figure 2. 5 Primary sequence alignment of HBO1 and its orthologs from various species all the way from yeast to human.

The sequence conservation and the crystal structure of yeast ESA HAT domain was used to design HBO1 HAT domain construct. The red region indicates evolutionary conservation

To determine if the minimal interacting domains of each protein were sufficient to interact in solution, each of these separately purified proteins were mixed together with one protein in excess and ran on a Superdex 200 size exclusion column. As expected, the HBO1 and JADE1 complex co-migrated with excess HBO1 or JADE1 eluting at a higher retention volume (**Figure 2.6**). In this study, we have demonstrated that the minimal interacting domains of HBO1 and JADE1 were able to form a stable hetero dimeric complex consistent with a 1:1 globular protein complex according to the retention volume on the s200 column. In addition, the fact that the protein complex eluted at the right molecular mark outside of the void volume indicates that the protein complex we prepared was properly folded.

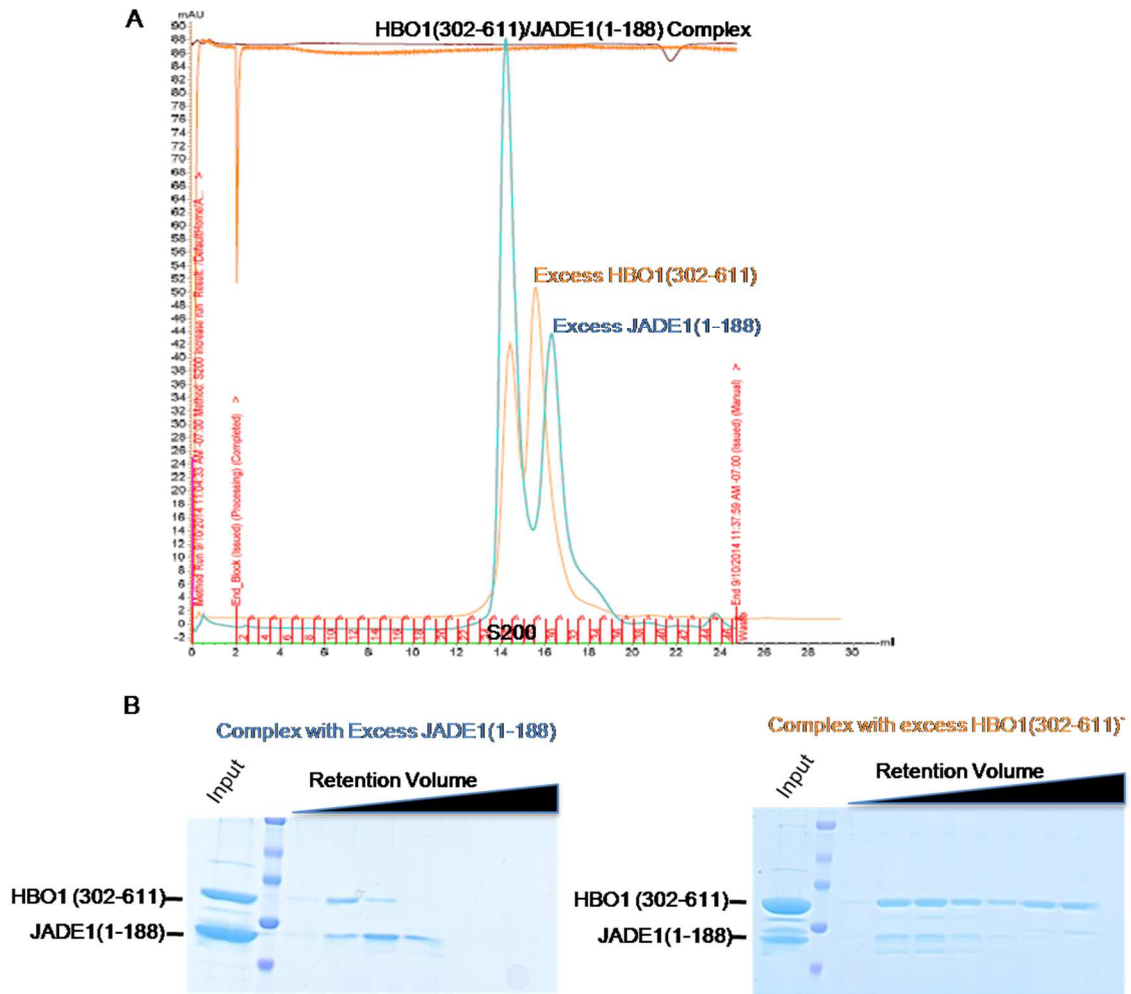


Figure 2. 6JADE domain I forms a complex with HBO1 HAT domain.

(A) S200 size exclusion chromatography run with HBO1 JADE1 complex with either HBO1 or JADE1 in excess (B) The protein sample of each fraction was resolved on SDS-PAGE gel

The purified HBO1 HAT domain (302-611) undergoes an N-terminal degradation over time (**Figure 2.7A**). The resulting degradation product was analyzed by mass spectroscopy, which indicated that HBO1 gets hydrolyzed around residue 329 (**Figure 2.8A**). Using Psi Pred secondary structure prediction(Jones, 1999), we were able to determine that the hydrolysis occurs at a predicted flexible region of the HAT domain (**Figure 2.8B**). We attempted to express and purify the hydrolyzed product. However, HBO1(329-611) was not soluble indicating that residues 302-329 of HBO1 play an important role in overall folding and stability of the HAT domain. Interestingly, when the HBO1 HAT domain (302-611) is purified with JADE1(1-188) as a complex, the N-terminal loop is protected and is able to remain as an intact protein, which results in a stable heterodimeric complex. This result reveals that when HBO1 and JADE1 are purified together as a stable complex, JADE1(1-188) protects the N-terminal region of the HBO1 HAT domain from degradation. In addition, HBO1 (302-611) and the HBO1(302-611)/JADE1(1-188) complex were used for a Thermal Shift Assay (using Sypro Orange binding as a readout for protein unfolding) to test their overall stability by measuring their apparent melting temperature. This study revealed that JADE1 (1-188) binding to the HBO1 HAT domain stabilized the HAT domain by $\sim 5^{\circ}\text{C}$ (Figure 2.7B). As a positive control, we demonstrated that the addition of acetyl-CoA increased the apparent melting temperature of the HBO1 HAT domain by $\sim 3^{\circ}\text{C}$ suggesting that we have a well-folded HAT domain that is able to bind cofactor (data not shown).

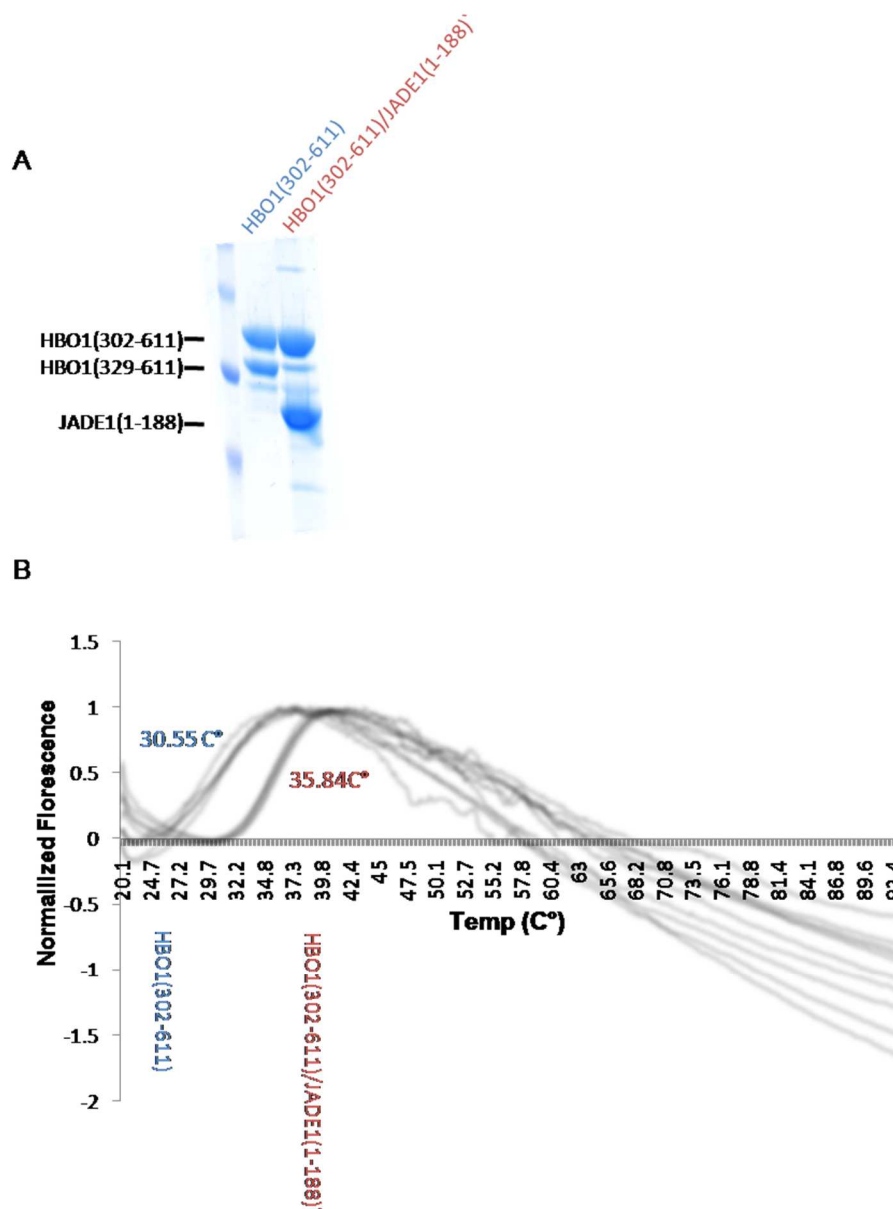


Figure 2. 7JADE1 projects the N-terminal end of HBO1 HAT domain.

(A) HBO1(302-611) and HBO1(302-611)/JADE1(1-188) complex resolved on SDS-PAGE gel (B) Thermal shift assay of HBO1(302-611) in blue and HBO1(302-611)/JADE1(1-188) complex in red

Database: UP081514_SBACecoliIgContBFR_Cust15.fasta
 Protein ID: cs/C00053/C00053_CUST
 Monoisotopic Mass: 37,022.77

Source: MarmorsteinR-15-G020-HBO1-30Kfrag-QEplus
 Protein Name: CUST JHan HBO1 sequence
 Amino Acid Coverage: 217/316 = 68%

1 316

1 GSEFELTSEY DDLFRRAQA RASEDLEKLR LQGQITEGS IKTIAPGRY ELDTWYHSPY PEEYARLGR L YNCEFC LKYM KSQTILRRHM

91 AKCVNKHPPG DEIYRKGSI VFEVDGKKN IYCC LCLLA KFLDHKTL YDVEFFLFYV MTEADNTGCH LIGYFSKES SFLIVIVSCI

181 LTIPQYRQG YGNLIDFSY LLSKVEEKVG SPERPLSDLG LISYRSYWK VLLRYLR FQ GKEISIKEIS QETAVIPVDI VSTLQALCNL

271 KYWKGKHLVL KRQDLIDEWI AKEAKRSNS KTDPSCLKW TPKKGT

* Site of hydrolysis

35

Figure 2. 8HBO1 is hydrolyzed at the flexible region in the HAT domain

(A) LC-MS/MS analysis of the degraded fragment of HBO1 (B) Psi Pred secondary structure prediction around the site of degradation

2.3.4 Domain I of JADE is sufficient for activation of HBO1 acetyltransferase activity towards H4 peptide

Having demonstrated that JADE1 (1-188) and the HBO1-HAT domain form a stable complex, we next set out to determine the catalytic contribution of JADE1(1-188) to HBO1 acetylation activity towards an H4 peptide. To do this, we titrated JADE1(1-188) up to 10-fold molar excess to HBO1(302-611) and tested for acetyltransferase activity using a radioactive-based acetyltransferase assay. These studies demonstrate that the recombinant HBO1 HAT domain harbors acetyltransferase activity towards H4 peptide, and that this activity increases in a dose response manner upon titration of JADE1 domain I (**Figure 2.9**). The size-exclusion chromatography run of HBO1 and JADE1 suggested that the complex has a stoichiometry of 1 to 1. To confirm this stoichiometry, we created the fusion construct JADE1(1-188)-HBO1(302-611) to force a 1:1 stoichiometry and test if additional JADE1(1-188) further stimulates catalytic activity of the fusion protein. The fusion construct was created by cloning both fragments into the same vector, expressing as a single polypeptide that is purified in a similar manner as HBO1(302-611). In agreement with the 1:1 JADE1(1-188)/HBO1(302-611) stoichiometry, we observed that while the fusion construct showed much increased activity compared to HBO1 (302-611) alone, it was not stimulated by titration of excess JADE1(1-188) (**Figure 2.9**). Although crystallographic information on the complex may be necessary to fully capture the multimerization state of the complex, this data is consistent with a 1:1 JADE1(1-188)/HBO1(302-611) stoichiometry.

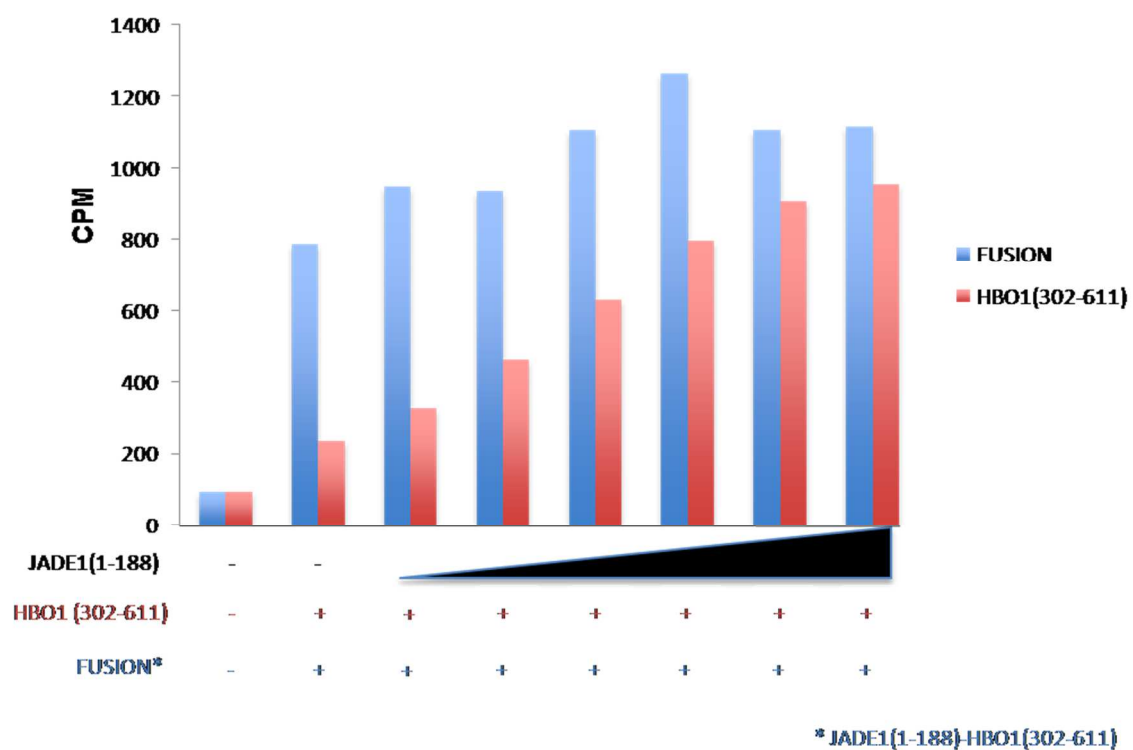


Figure 2. 9HBO1 HAT domain responses to addition of JADE Domain I in acetyltransferase activity

Acetyltransferase activity of HBO1(red) and JADE1-HBO1 fusion(blue) done with H4 peptide as the substrate. JADE is titrated up to 10 fold molar excess.

In addition, to further analyze the catalytic contribution of JADE1(1-188) to HBO1 HAT domain acetyltransferase activity, we have determined the steady state parameters, V_{\max} and K_m using substrate saturation curves with A-CoA (~100uM) and H4 peptide (~1mM) (Figure 2.10). Here, we demonstrated that the minimal catalytic core HBO1(302-611)/JADE1(1-188) of the HBO/JADE HAT complex is sufficient for interaction as well as catalysis. Specifically, when HBO1 HAT domain was tested for its acetyltransferase activity with the H4 peptide as the substrate, JADE1(1-188) affected both K_m and V_{\max} on A-CoA by two- and three-fold increase, respectively. However, JADE1(1-188) only increased the V_{\max} on H4 peptide by three-fold while not affecting the K_m . The fact that JADE1 did not affect the K_m while increasing the V_{\max} on H4 peptide suggests that JADE1 is able to increase the enzymatic rate of HBO1 without contributing to the overall affinity of the HBO1 HAT domain and H4 tail. Although a further study is necessary to fully understand how JADE1 activates HBO1 for acetyltransferase activity, our data suggests that JADE1 affects the enzymatic rate of HBO1 by modulating the active site either through stability or allostery.

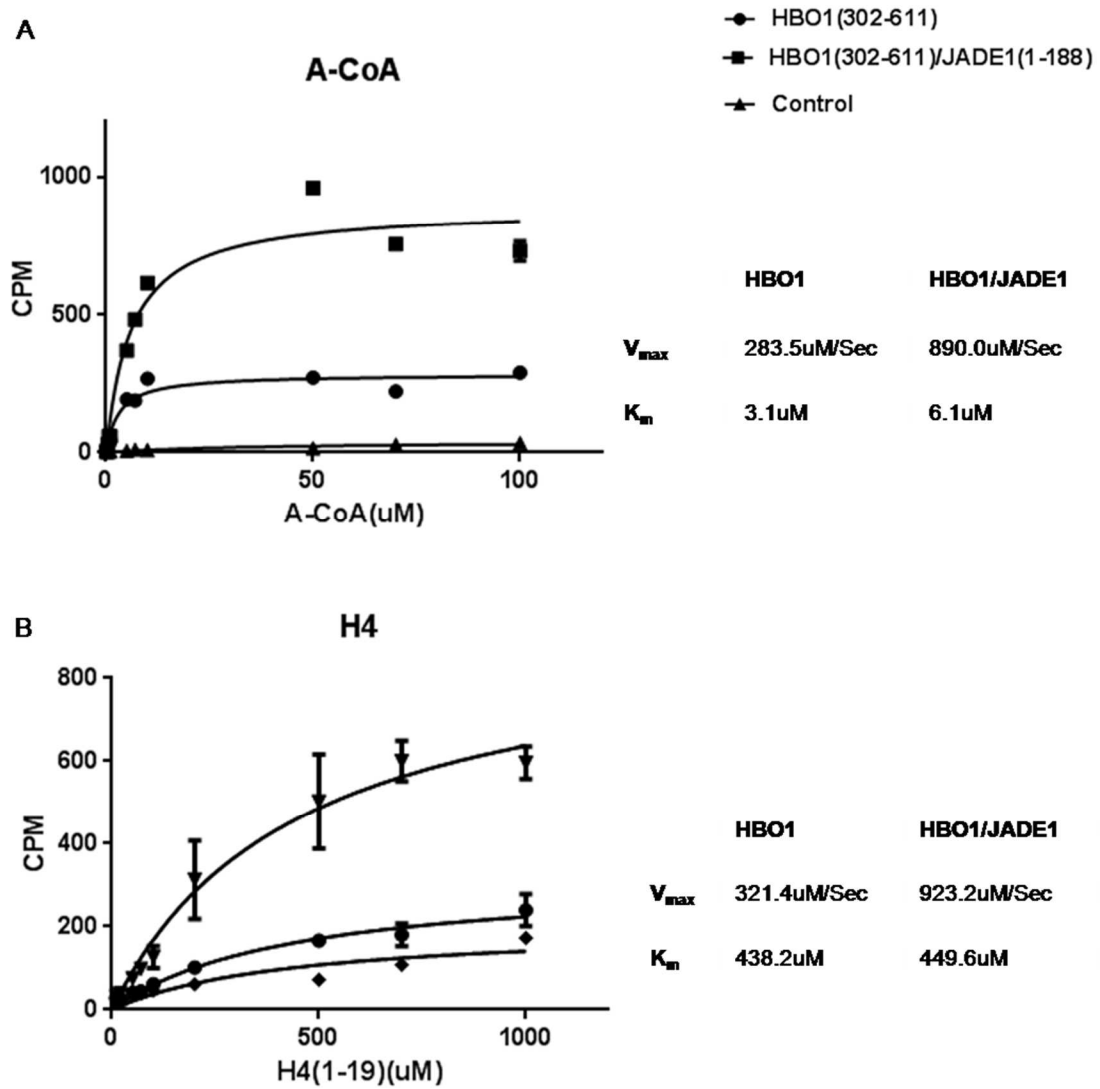


Figure 2. 10JADE1 Domain I (1-188) is able to stimulate activity of the isolated HBO1 HAT domain towards H4 peptides

Saturation curve of A-CoA and H4 of the HBO1(302-611) and HBO1(302-611)/JADE1(1-188) using the Michaelis-Menten kinetic model. The output of the assay is displayed as counts per minute (CPM) (A) A-CoA (B) H4 peptide (1-19)

2.4 Discussion

HBO1 functions in a multiprotein HAT complex and has been shown to be the main player in H4 acetylation(Lalonde et al., 2013). Previous studies have demonstrated that other subunits of the complex contribute to H4 acetyltransferase activity of HBO1(Avvakumov et al., 2012). There are many components that may contribute to the overall activity of HBO1, such as chromatin recognition domain within the complex that allows the complex to be localized to the correct region of chromatin. However, the relative contributions of subunits of the HBO1 complex to HBO1 catalytic activity has not been previously dissected. In this study, we demonstrate that the JADE subunit, Domain I in particular, of the HBO1 complex plays the most significant role in HBO1 H4 acetyltransferase activity. We also find that JADE protects the N-terminal region of the HAT domain, which seems to be important for overall stability of the HAT domain. This data is consistent with previous studies demonstrating that HBO1 can complex either with JADE or BRPF leading to either H4 or H3 tail acetylation, respectively(Lalonde et al., 2013).

A recent crystal structure of the HAT domain of HBO1 in complex with an interacting peptide of BRPF2 reveals that BRPF2 wraps around the N-terminal end of the HAT domain(Tao et al., 2017). This observation is in agreement with our hypothesis that JADE1 might be interacting and protecting the N-terminal end of the HAT domain. The authors of the HBO1-HAT/BRPF2 complex also demonstrate that BRPF2 binding to the N-terminal region of the HBO1-HAT domain confers H3 acetylation activity, and in analogy we propose that JADE binding to N-terminal region of the HBO1-HAT domain confers H4 acetylation activity.

2.5 Materials and Methods:

2.5.1 Full-length HBO1 HAT complexes

Flag-HBO1, HA-JADE1/3, HA-Eaf6, and HA-ING4 in pVL vectors were provided by Jacques Côté. Baculovirus Sf9 insect cells were used to prepare 4-protein and 2-protein HBO1 complexes and

FLAG-HBO1. pVL-FLAG-HBO1, pVL-HA-JADE3, pVL-HA-Eaf6, and pVL-HA-ING4 vectors were co-infected for the 4-protein complex, and pVL-FLAG-HBO1, pVL-HA-JADE3 were co-infected for the 2-protein complex. Cells were harvested at 48 hours by centrifugation at 3500 RPM. Cells were lysed by sonicating in lysis buffer (20mM Tris(pH 8) 500mM NaCl, 1mM BME, 0.1mg/ml PMSF/ c0mplete, EDTA-free protease inhibitor cocktail tablets). The post-lysis sample was spun down at 20,000 RPM for 30 minutes and the soluble fraction of the post-lysis sample was applied to Anti-FLAG Resin (Sigma Aldrich) and incubated for an hour at 4°C. Proteins were eluted with 500ug/ml 3XFLAG peptide (Sigma Aldrich) in the lysis buffer.

2.5.2 Suprose-6 size exclusion chromatography.

3XFLAG peptide eluted proteins were concentrated with 50KDa cutoff concentrators (Millipore) and injected onto aSuprose-6 size-exclusion column (GE) with a column flow rate of 0.2ml/min. Elutions were fractionated and collected in 1 ml fractions.

2.5.3 JADE1/2/3 N-terminal sequence alignment

Sequence alignment for JADE1/2/3 was carried out using ClustalW2(Sievers et al., 2011) and was further formatted using ESPript (3.0) for more optimal sequence homology of JADE homologs(Gouet et al., 2003).

2.5.4 Recombinant HBO1 and JADE1 protein production in bacteria

DNA encoding HBO1 and JADE1 protein constructs were generated by PCR amplification of the corresponding DNAs, which were cloned into pRSF or pDB.His.MBP vectors using SacI/NotI sites with a cleavable N-terminal affinity tags (GST-,6XHIS-,MBP-). Cells containing these cloned constructs were grown and expressed using BL21(DE3) cells in LB. Cells were induced for protein over-expression at an OD ~0.6 with 0.5mM IPTG for 16-18 hours at 18 °C. Cells were harvested by centrifugation at 4000 RPM and lysed by sonicating in lysis buffer (20mM Tris(pH 8) 500mM NaCl, 5mM BME, 0.1mg/ml PMSF). The lysed samples were spun down at 20,000 RPM

for 30 min. and the soluble fraction of the lysed samples were applied to either, Pierce Glutathione Agarose resin(Thermo), Amylose resin (NE Biolabs) or Ni-NTA resin (Thermo) for batch binding at 4°C for 1 hour. Samples were washed with 20 column volumes (CV) of wash buffer (20mM Tris(pH 8), 500mM NaCl, 5mM BME) then eluted with eluant with either 30mM reduced L-glutathione(GST), 20mM maltose monohydrate(MBP), or 300mM Imidazole(6XHIS). Proteins were further purified to homogeneity by either HiTrapQ(GE Healthcare) anion exchange for JADE1 constructs or HiTrapHP(GE Healthcare) cation exchange for HBO1 and fusion constructs. Ion exchange chromatography was performed using a NaCl gradient from 100mM to 1M over 30 CV. To ensure the proper fold of recombinant proteins, they were subjected to size exclusion chromatography (SEC) using a Superdex 200 column in 20mM Tris(pH 8), 500mM NaCl, 5 mM BME.

2.5.5 Histone acetyltransferase(HAT) activity assay

HBO1 HAT activity was measured using a previously described HAT assay with ¹⁴C labeled Ac-CoA (50-60 mCi/mmol; Moravsek)(Yan et al., 2002). A time course for the reaction was initially performed to determine the linear range of enzyme activity at an enzyme concentration of 200nM(HBO1)/1uM(HBO1-HAT complex) and saturating concentrations of AcCoA (300uM) and H4 peptide (1mM). The HAT reaction was performed using a buffer containing 20mM Tris (pH 7.5), 500mM NaCl, 0.25mg/ml BSA for 1 hour at 20°C in a volume of 50uL. 20uL of the reaction was spotted on P81 filter paper. The positively charged and C¹⁴-labeled H4 peptide was captured on the filter paper, while free ¹⁴C-labeled Ac-CoA was washed away. The paper was analyzed using Liquid Scintillation(Berndsen and Denu, 2005). The amount of ¹⁴C-labeled H4 peptide bound to the paper is directly proportional to the amount of acetyl group transferred from the cofactor to peptide, thus the data was used to evaluate HBO1 acetyltransferase activity(Berndsen and Denu, 2005). All reactions were done in duplicate. The output of the enzyme was plotted as Counts Per Minute(CPM).

Chapter 3 -The scaffolding protein JADE1 physically links the acetyltransferase subunit HBO1 with its histone H3/H4 substrate

This research was performed in a collaboration with Dr. Catherine Lachance, and Dr. Jacques Côté of the Laval University Cancer Research Center. Dr.Catherine Lachance, and Dr. Jacques Côté carried out the cellular experiments in section 3.3.7. Repeated with permission from Han, J. *et al.* The scaffolding protein JADE1 physically links the acetyltransferase subunit HBO1 with its histone H3-H4 substrate. *J Biol Chem***293**, 4498-4509, doi:10.1074/jbc.RA117.000677 (2018).

3.1 Abstract

The human HBO1 enzyme regulates DNA replication, cell proliferation and development in the context of a multi-protein histone acetyltransferase (HAT) complexes containing ING4/5, MEAF6 and scaffolding proteins JADE1/2/3 or BRPF2/3 to acetylate histone H4 H4K5/8/12 or H3K14, respectively. Within the four-protein complex, the JADE protein has been demonstrated to be the determinant for histone H4 substrate specificity of the HBO1 HAT complex. We carried out *in vitro* studies with recombinant proteins to dissect the specific regions and activities of HBO1 and JADE1 that mediate histone H4 acetylation by the HBO1 HAT domain. We find that JADE1 increases the catalytic efficiency of HBO1 acetylation of a H3/H4 substrate by about 5-fold through a N-terminal 21-residue HBO1 and Histone Binding Domain (HHBD) and a nearby second Histone Core Binding Domain (HCBD). We also demonstrate that HBO1 contains an N-terminal Histone Binding Domain (HBD) that makes additional contacts to H3/H4 independent of JADE1, interactions with histones and the HBO1-HBD does not significantly contribute to the overall HAT activity of HBO1. Cellular experiments employing JADE1 deletions recapitulate these *in vitro* interactions and their roles in HBO1 histone acetylation activity. Together, these studies demonstrate that the N-terminal region of JADE1 functions as a platform to bring the catalytic HBO1 subunit together with its cognate H3/H4 substrate for histone acetyltransferase activity.

3.2 Introduction

Histone acetylation, a covalent modification on lysine residues of histones, predominantly in the N-terminal tail regions, regulates local and global chromatin dynamics—thus playing an important role in modulating DNA accessibility and gene expression in all eukaryotic organisms. Generally, hyperacetylation and hypoacetylation are observed in transcriptionally active and inactive regions of the chromatin, respectively (Havasi et al., 2013; Kornberg and Lorch, 1999; Sapountzi and Cote, 2011; Strahl and Allis, 2000; Thomas and Voss, 2007). Histone acetylation has been shown to promote transcription in at least two ways. First, acetylation neutralizes the

lysine positive charge, thus altering its physiochemical properties and making the local chromatin environment more accessible for gene transcription activation(Sapountzi and Cote, 2011). Second, lysine acetylation generates a docking recognition sites for various gene-activating proteins containing acetyl-lysine recognition domains such as bromodomains(Havasi et al., 2013; Jenuwein and Allis, 2001; Peterson and Laniel, 2004; Taverna et al., 2007; Turner, 2000). Therefore, the tight regulation of acetylation on lysine residues within histones, as well as the enzymes that mediate these modifications, are critical to maintain normal gene expression and regulation.

The enzymes that mediate histone acetylation are histone acetyltransferases (HATs), while the enzymes that convert acetyl-lysine residues within histone back to lysine are histone deacetylases (HDACs)(Allis et al., 2007). HDACs are grouped into four classes according to their functional properties and sequence conservation, which fall into two conserved structural folds (classes I, II, IV and the class III sirtuins). HATs show considerably more sequence and structural diversity than HDACs, forming at least five different families, Gcn5/PCAF, MYST, p300/CBP, Rtt109 and HAT1. HATs typically function within multiprotein complexes *in vivo* where their substrate specificities and activities are modulated by the other subunits of the respective HAT complexes(Lalonde et al., 2013; Lalonde et al., 2014). Human HBO1 (also referenced as KAT7 or MYST2), a member of the MYST family, regulates DNA replication(Doyon et al., 2006; Miotto and Struhl, 2008), cell proliferation and development(Avvakumov et al., 2012). Consistent with its role in gene expression, the HBO1 HAT complexes are localized at the transcription start sites and coding regions of active genes in the genome(Saksouk et al., 2009), and is responsible for the majority of H4(Doyon et al., 2006; Foy et al., 2008; Miotto and Struhl, 2010) and H3K14(Feng et al., 2016; Kueh et al., 2011) acetylation. HBO1 has also been shown to play more specific roles in activating the expression of key genes in development and embryonic patterning(Kueh et al., 2011). The aberrant activity of HBO1 has been correlated with several cancers. HBO1 has been shown to be over-expressed in MCF7 and Saos-2 established cancer cell lines and in a subset of human primary cancers such as carcinomas of testis, breast, and ovary(Duong et al., 2013;

lizuka et al., 2009). Finally, a HBO1 catalytic defective mutant inhibits MCM complex loading onto the origin, indicating that HBO1 functions as a DNA replication co-activator in addition to its transcriptional roles(Havasi et al., 2013; Miotto and Struhl, 2008, 2010).

JADE1 has been demonstrated to act as a platform to assemble the tetrameric HBO1 HAT complex for histone H4 acetylation, with a region of JADE1 N-terminal to the JADE1 PHD fingers (called region I) shown to recruit HBO1, and a region of JADE1 C-terminal to the JADE1 PHD fingers (called region II) shown to recruit the ING/MEAF6 subcomplex(Avvakumov et al., 2012). It has also been shown that a modified tetrameric complex, in which BRPF replaces JADE has specificity for H3K14 acetylation(Lalonde et al., 2013). These studies highlight the importance of BRPF and JADE subunits in dictating histone substrate specificity. While a recent study has revealed the molecular basis for how BRPF mediates H3K14-specific acetylation by HBO1(Tao et al., 2017), the molecular basis for how JADE potentiates the H4 specific activity of HBO1 is not known. Given this gap in knowledge, we set out to dissect the specific regions and activities of JADE1 that mediate histone H4 acetylation by the HBO1 HAT complex. We find that JADE1 contains specific HBO1 and histone binding regions that serves to increase the catalytic efficiency of HBO1 acetylation of histone H4. Implications of these studies for the differential regulation of HBO1 by JADE1 and BRPF1 paralogs are discussed.

3.3 Results

H4 tails are a part of H3/H4 histone heterodimers and heterotetramers in the cell and are never found alone in the cell. For this reason, our subsequent studies of HBO1 complex activity employed H3/H4 complexes as substrates. To better understand the substrate selection, we prepared 4 different constructs of the full length and tailless (TL) histones; H3/H4, H3(TL)/H4, H3/H4(TL), H3(TL)/H(TL) (**Figure 3.1**). Each of these histone pieces was prepared as indicted in the experimental procedure section in chapter 3. From this experiment we were able to see that the main target of the HBO1 on the H3/H4 was the lysine residues on the tail. More importantly

JADE mainly stimulated HBO1's activity toward H4 tails of the histone. In this chapter, we will dissect the roles of two newly identified N-terminal JADE domains that influence substrate recruitment for substrate specificity of the HBO1 HAT complex.

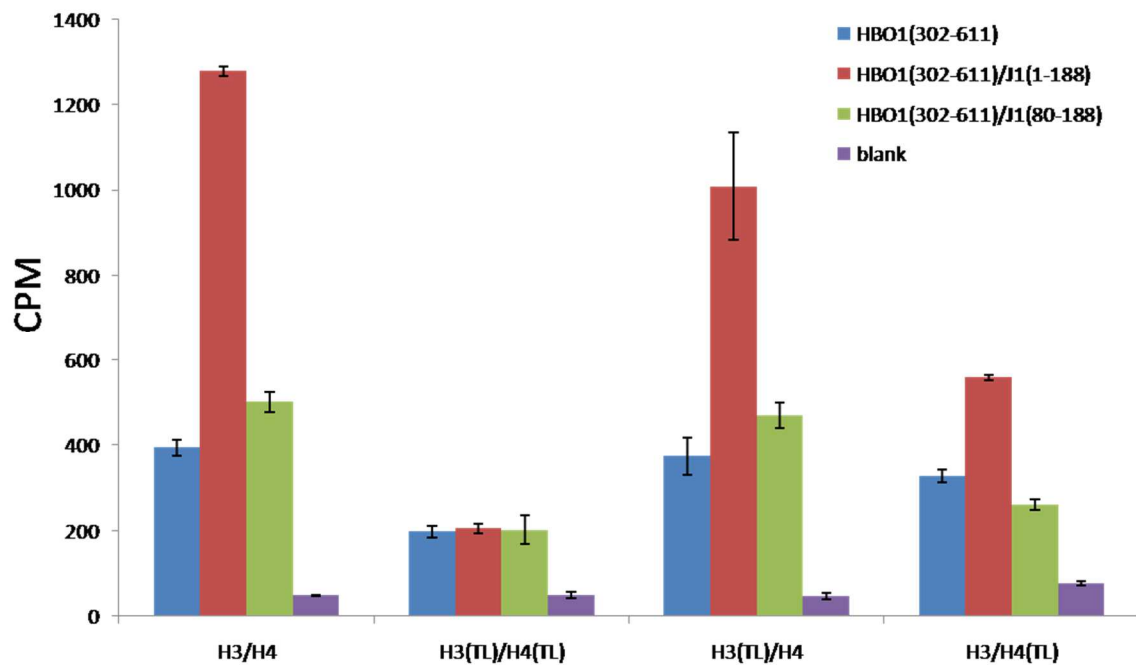


Figure 3. HBO1 acetyltransferase activity on different histone substrates

Acetyltransferase activity of HBO1 with 4 different histone constructs and its response to addition of 2 different JADE1 constructs. The output of the assay is displayed as counts per minute (CPM)

3.3.1 A 21-residue segment of JADE1 directly contacts the HBO1 HAT domain and histone substrate to facilitate acetylation of free histones

It was previously demonstrated that an N-terminal region of JADE1 between residues 90-199 participated in HBO1 catalytic activity(Avvakumov et al., 2012). This region of JADE1 contains sequence conservation within residues 115-179 of yeast to human proteins associated with ING/MYST-containing HAT complexes(Avvakumov et al., 2012; Lalonde et al., 2013). We also noted an additional region of sequence conservation among the JADE proteins just N-terminal to this region (**Figure 3.2A**). To map the region of the JADE1 N-terminus that directly interacts with the HBO1-HAT domain, we used pull down experiments with the HBO1-HAT and N-terminal truncation constructs of JADE1 (**Figure 3.2B**). These experiments revealed that MBP-JADE1(1-188) and MBP-JADE1(60-188) were able to pull down the HBO1-HAT, while MBP-JADE1(80-188) was not. These data revealed the importance of residues 60-80 of JADE1 for the direct interaction with the HBO1-HAT. To further narrow down the key residue(s) within this 21-residue segment critical for the interaction with the HBO1-HAT domain, we introduced triple alanine mutations spanning residues 60 to 80 within the context of MBP-JADE1(1-188). These studies revealed that each of the mutants resulted in no detectable HBO1-HAT pull down (**Figure 3.2C**). The observation that each of the JADE1 mutants disrupted HBO1-HAT interaction suggested that either the entire JADE1(60-80) domain makes important contacts to the HBO1-HAT or that the domain contains a binding surface and tertiary structure that are both important for HBO1-HAT interaction. To more quantitatively assess the binding of the JADE N-terminal region to the HBO1-HAT, we performed Isothermal Titration Calorimetry (ITC) using various constructs of JADE1. The ITC results were consistent with the pull-down data, demonstrating that JADE1(60-188) showed significant binding to the HBO1-HAT with a K_d of $\sim 2.4\mu\text{M}$ and a stoichiometry of $\sim 1:1$, while JADE1 (80-188) showed no detectable binding (**Figure 3.2D**). Strikingly, a peptide of residues 55-85 of JADE1 showed binding with a comparable dissociation constant ($K_d = 4.18\mu\text{M}$) to JADE1(60-188) demonstrating that residues 60-80 of JADE1 are essential for HBO1-HAT binding.

We next asked if residues of 60-80 of JADE1 are also able to interact with histones to potentially bridge the HBO1-HAT acetylation of histones. To do this, we carried out pull-down studies of MBP-JADE1 N- and C-terminal deletion constructs with HBO1-HAT plus H3/H4 (Figure 3.2B). These studies revealed that only JADE1 constructs harboring residues 60-80 of JADE1 were able to interact with both the HBO1-HAT and H3/H4, confirming that JADE1 residues 60-80 bridge HBO1-HAT/histone interactions. Notably, JADE1 constructs starting at residue 60 and extending to residue 188, showed more robust pull down with HBO1-HAT and histones than JADE1 constructs harboring only residues 60-80, suggesting that additional regions of JADE1 between residues 108 and 188 also contribute to HBO1/histone interactions (**Figure 3.2B**). Based on the HBO1-HAT and histone binding properties of residues 60-80 of JADE1, we refer to it as the HBO1 and Histone Binding Domain (JADE1-HHBD).

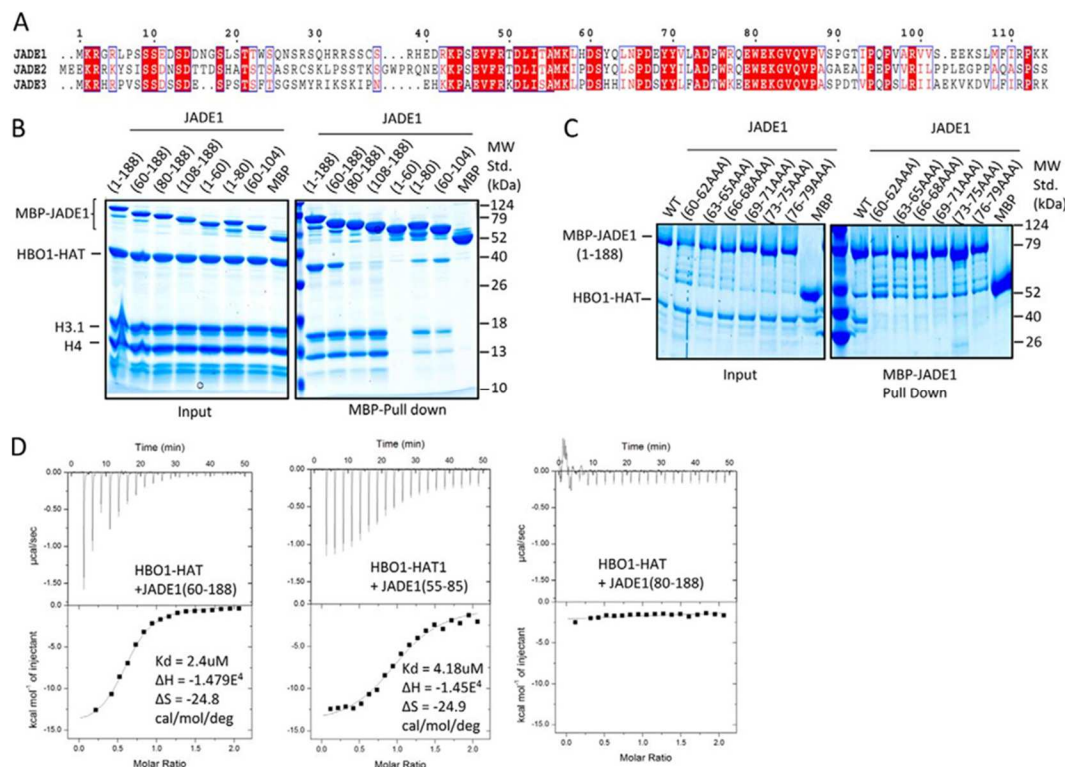


Figure 3. 2An N-terminal region of JADE1 binds HBO1.

(A) sequence alignment of the N-terminal region of JADE1/2/3 paralogs, with the highest degree of conservation shaded in red and conservative substitutions colored in red. The HBO1 and HHBD are highlighted. (B) MBP-JADE1 pulldowns with various N-terminal and C-terminal deletion constructs of the HBO1-HAT domain and H3/H4 complex with results resolved on SDS-PAGE. MW Std., molecular weight standard. (C) MBP-JADE1 pulldowns of the HBO1-HAT with JADE1 triple alanine mutations with results resolved on SDS-PAGE. (D) ITC studies of JADE1 constructs titrated into the HBO1-HAT, showing the heat profile (top) and the calculated binding isotherm (bottom). Thermodynamic data calculated from this data are shown at the right.

We then asked if the minimal region of JADE1 that facilitates HBO1 and histone binding is sufficient to potentiate HBO1-HAT activity toward histone tail acetylation. To do this, we titrated JADE1 (55-85) and JADE1(1-188) into the HBO1-HAT and H3-H4 complex and assayed for

histone acetyltransferase activity (**Figure 3.3**). This analysis revealed that titration of JADE1(55-85) in vast excess resulted in only marginal effects on HBO1 histone acetyltransferase activity, while titration of JADE1(1-188) showed significantly more potentiation of HBO1 histone acetyltransferase activity, to a level of about 6-fold relative to no added titrant. Taken together with the previous data showing that residues 60-80 of JADE1 bridges HBO1-HAT-histone interaction, these data demonstrated that residues 60-80 of JADE1 contribute to but are not sufficient to mediate HBO1 acetylation of histones.

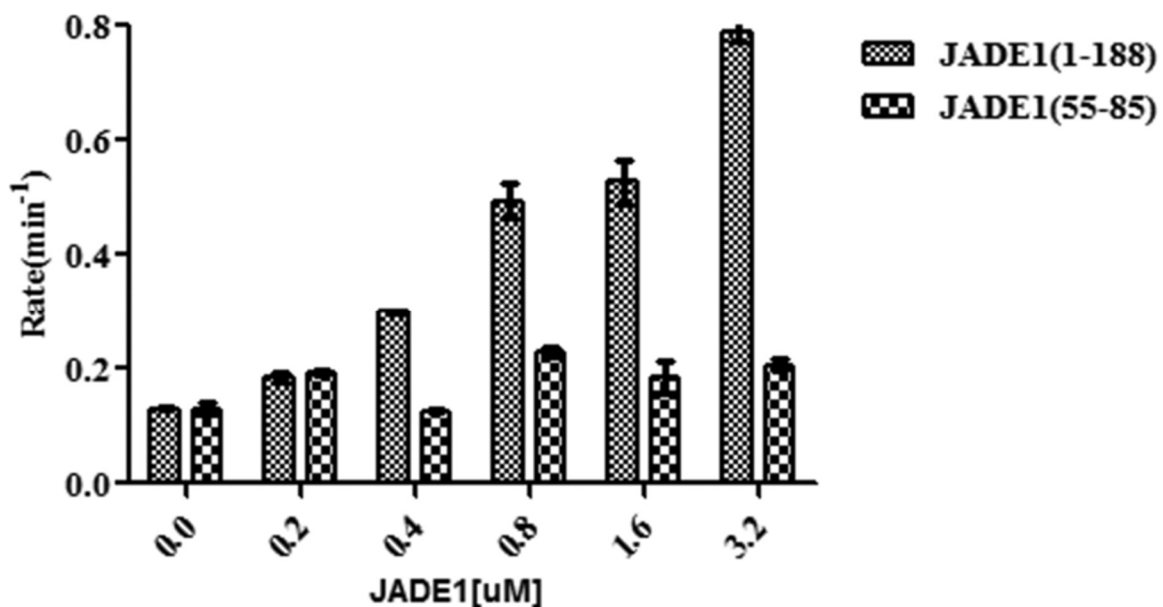


Figure 3. 3JADE1 requires an additional N-terminal region for H3/H4 binding and HBO1 HAT potentiation.

Shown is the titration of JADE1 constructs (55–85 and 1–188) from 0- to 16-fold molar excess of HBO1 HAT domain tested for acetyltransferase activity on the H3/H4 complex. Radioactive counts are converted to enzyme rate as described under “Experimental procedures.”

3.3.2 JADE1 contains a second histone binding domain that contributes to HBO1 acetylation of histone H4 substrate by anchoring the HBO1/JADE1 complex to the histone core.

Inspection of the pull-down experiments of Figure 3.2B reveals that an additional region of JADE1, within residues 108-188, interacts with histones but not the HBO1-HAT and the histone acetyltransferase activity assay of Figure 3.3 further suggests that regions within JADE1 residues 80-188 are also required to fully potentiate HBO1-HAT histone acetyltransferase activity. Based on these studies, we carried out further studies to define the regions of JADE1 and histones that

mediate this interaction. To specifically probe the requirement for the histone tails for JADE1 interaction, we carried out MBP-JADE1(60-188) pull down with H3/H4 with either full-length or tailless H3 and/or H4 subunits. These studies revealed that JADE1(60-188) pulled down all of the histone complexes, including the completely tailless complexes (H3(TL)-H4(TL)) (**Figure 3.4A**). These pull-down experiments demonstrated that the primary JADE1(60-188) binding site exists on the core of the H3/H4 complex.

Previous studies indicate that JADE1 facilitates HBO1 H4-specific tail acetylation (Lalonde et al., 2013). For this reason, we asked if JADE1 makes an additional direct or HBO1 dependent contact with the H4 tail. To test this, we prepared GST tagged H4 tail 1 to 19 and performed a GST pull down with JADE1 (1-188) in the presence of the HBO1-HAT. This experiment revealed that HBO1-HAT is unable to interact directly with the isolated H4 histone tail in the absence or presence of JADE1(1-188) (**Figure 3.4B**), consistent with the conclusion that JADE1 histone core binding is required for HBO1-HAT acetylation of the histone H4 tail. We refer to the domain of JADE1 that mediates H3/H4 core binding as the Histone Core Binding Domain (JADE1-HCBD).

MBP pull downs with JADE1 deletion constructs and H3/H4 (**Figure 3.2B**) suggests that JADE1 contains 2 histone binding sites, JADE1(60-80), JADE1(104-188). To more quantitatively evaluate the histone binding properties of these two regions of JADE1, we performed Isothermal Titration Calorimetry (ITC) with H3/H4 using various constructs of JADE1. The ITC data showed that JADE1(60-104) and JADE1(80-188) bind H3/H4 with comparable dissociation constants of 59.17uM and 52.52uM, respectively (**Figure 3.4C**), while JADE1 (60-188), containing both histone binding sites, showed H3/H4 binding with a dissociation constant of about 2-fold higher of 29.41uM (**Figure 3.4C**). These studies indicate that the HHBD and HCBD regions of JADE1 bind comparably and additively to H3/H4.

We also carried out MBP-JADE(1-188) pull down studies with H3/H4 (with or without tails) in the presence or absence of the HBO-HAT, revealing that the histone binding properties of JADE1(1-188) are independent of HBO-HAT binding (**Figure 3.4D**).

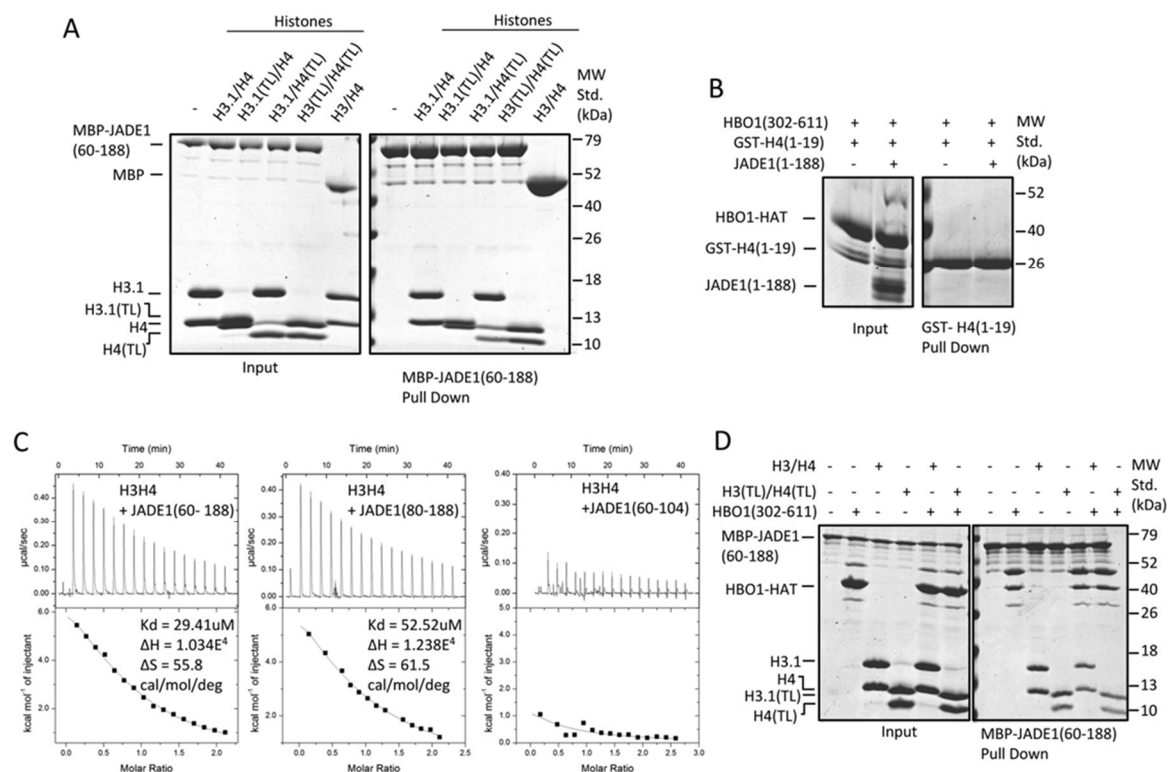


Figure 3. 4The additional JADE1 N-terminal HCB specifically binds the histone core region.

(A) MBP-JADE1 pulldowns of four different H3/H4 complexes (H3/H4, H3(TL)/H4, H3/H4(TL), H3(TL)/H4(TL)) with results resolved on SDS-PAGE. MW Std., molecular weight standard. (B) GST-H4(1-19) pulldowns of HBO1-HAT in the presence and absence of JADE1(1-188) with results resolved on SDS-PAGE. (C) ITC studies of JADE1 constructs titrated into the H3/H4 complex showing the heat profile (top) and the calculated binding isotherm (bottom). Thermodynamic data calculated from these data are shown at the right. (D) MBP-JADE1 pulldowns of HBO1-HAT and H3/H4 complex in the presence and absence of each other, with results resolved on SDS-PAGE.

3.3.3 JADE1-HCBD shows selectivity for free histone H3/H4 over H2A/H2B and nucleosomes

To further probe the histone binding specificity of JADE1-HCBD, we performed pull down experiments with MBP-JADE1(1-188) and histones H3/H4 and H2A/H2B. These studies demonstrated that although JADE1(1-188) interacts with histone H3/H4 in the presence or absence of the HBO1-HAT, it does not interact with histone H2A/H2B (**Figure 3.5A**). Moreover, if we carry out similar pull-down studies with a mixture of H3/H4 and H2A/H2B complexes, JADE1(1-188) specifically selects for H3/H4 over H2A/H2B, suggesting that JADE1-HCBD does not interact with the H3/H4 core through a nonspecific charge interaction (**Figure 3.5B**).

It has been previously demonstrated that the H3 tail is essential for the HBO1-HAT complex to interact with chromatin(Saksouk et al., 2009). JADE1 contains 2 PHD finger domains (PHD-Zn knuckle-PHD or PZP domain) C-terminal to the JADE-HCBD that recognize specific methylation states of the H3 tail and allow the HBO1 HAT complex to bind and acetylate nucleosomes(Avvakumov et al., 2012; Foy et al., 2008; Saksouk et al., 2009). Given this information, we wanted to further investigate if the JADE1a -HCBD can bind to H3/H4 when it is assembled into a nucleosome. Pull-down studies with the assembled nucleosome, revealed the absence of an interaction (**Figure 3.5C**), suggesting that the octamer or the DNA is excluding the JADE1-HCBD binding surface to H3/H4 (**Figure 3.5C**). To further substantiate these findings, we compared the acetylation activity of HBO1-HAT and HBO1-FL with or without JADE1(1-188) against H3/H4 and nucleosomes. These studies revealed that JADE1(1-188) potentiated the activity of HBO1-HAT and HBO1-FL against H3/H4 but not nucleosomes (**Figure 3.5D**). This data suggests that other regions of JADE1 are required for HBO1 targeting to nucleosomes. This is consistent with previously reported data that the PZP, PHD1 and PHD2 domains of JADE1, located C-terminal to the JADE1 HHBD and HCBD domains, contribute to HBO1-mediated acetylation of nucleosomes(Avvakumov et al., 2012; Lalonde et al., 2013; Saksouk et al., 2009). In contrast, it appears that the HHBD and HCBD domains of JADE1 play an important role in

capturing free histones (H3/H4) during transcription (or replication) for further acetylation of the tails.

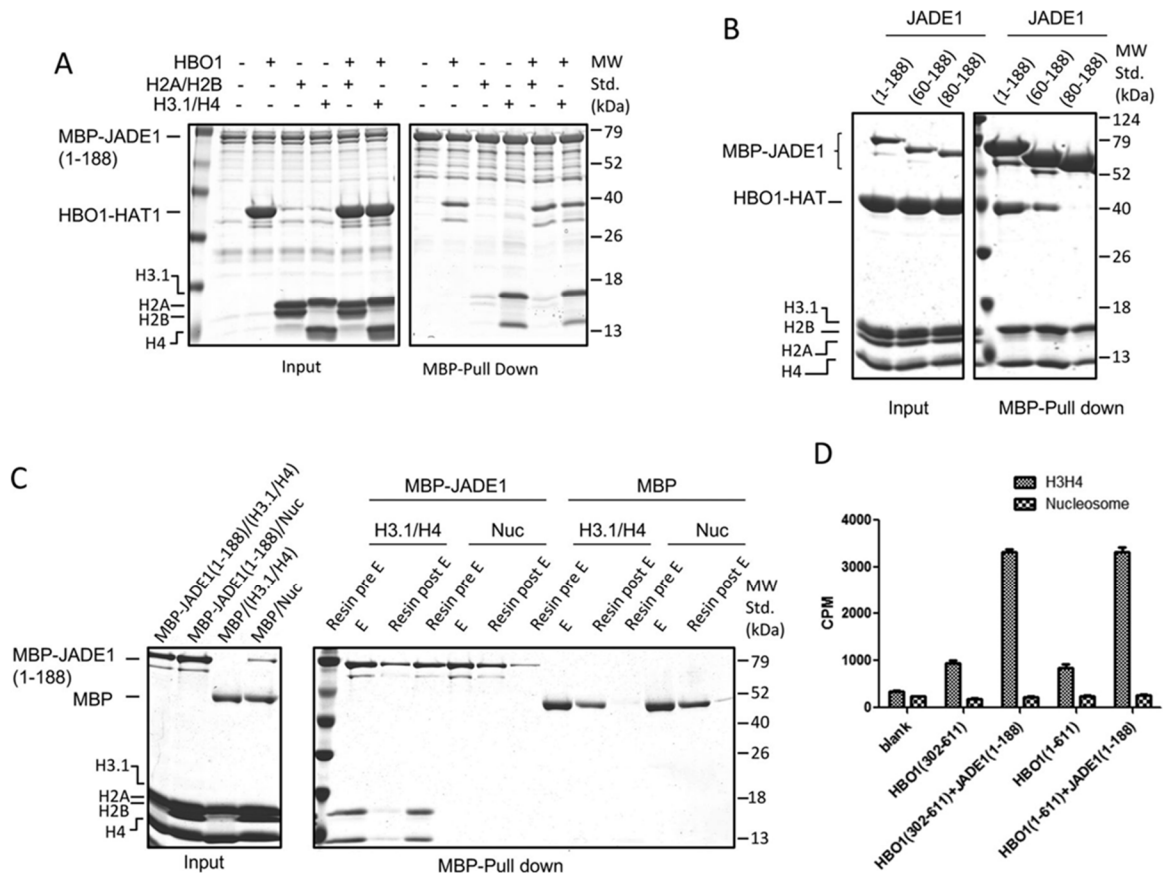


Figure 3. 5JADE1 selectively binds to H3/H4 over H2A/H2B and the nucleosome core particle.

(A) MBP-JADE1 pulldowns of HBO1 HAT and (H3/H4 or H2A/H2B) in the presence or absence of each other, with results resolved on SDS-PAGE. MW Std., molecular weight standard. (B) MBP-JADE1 pulldowns of HBO1 HAT and H3/H4, H2A/H2B and mixtures of the two with results resolved on SDS-PAGE. (C) MBP-JADE1 pulldowns of H3/H4 or nucleosome core particles with results resolved on SDS-PAGE. (D) activity comparison of HBO1 HAT and HBO1 full-length in the presence and absence of a saturating concentration of JADE1(1-188) as either H3H4 complex or nucleosome core particles as the substrate. The activity of these proteins on various substrates was compared using radioactive counts.

3.3.4 JADE1-HHBD and JADE1-HCBD bind independently to their respective partners, but cooperate to potentiate HBO1-HAT acetylation

After establishing that the HHBD and HCBD regions of JADE1 bind independently to their respective histone and/or HBO1-HAT binding partners, we set out to determine the contribution of these JADE1 regions for HBO1 acetyltransferase activity. We prepared four JADE1 deletion constructs spanning the HHBD and/or HCBD regions: 1-188(HHBD-HCBD), 60-188(HHBD-HCBD), 80-188(HCBD) and 1-80(HHBD). We assayed HBO1 HAT activity in the presence of 25-fold molar excess of each of these JADE1 constructs. This experiment revealed that only a JADE1 construct spanning both the HHBD and HCBD showed potentiating affects of HBO1-HAT activity. (**Figure 3.6A**). Moreover, titration with separate fragments of JADE1 1-80(HHBD) and JADE1 80-188(HCBD) did not potentiate HBO1-HAT acetylation activity (**Figure 3.6A**). These results demonstrate that the JADE1-HHBD and HCBD regions work cooperatively to potentiate HBO1 HAT activity and that these two regions of JADE1 must be connected to mediate this activity.

To more quantitatively assess the contribution of JADE1 to HBO1-HAT catalytic activity, we carried out bisubstrate kinetics. Due to a significant chemical acetylation that occurs on the core of histones at high concentration, we were not able to carry out a full Michaelis-Menten plot. Instead, we measured the catalytic efficiency (K_{cat}/K_m) using the slope of the linear range in the Michaelis-Menten plot. We found that JADE1(1-188) increased the catalytic efficiency of HBO1 by ~5 fold (**Figure 3.6B**). Together, this data demonstrates the importance of JADE1 in contributing to the overall catalytic activity of the HBO1 HAT complex by anchoring the complex to the substrate and by properly orienting the HBO1 HAT domain.

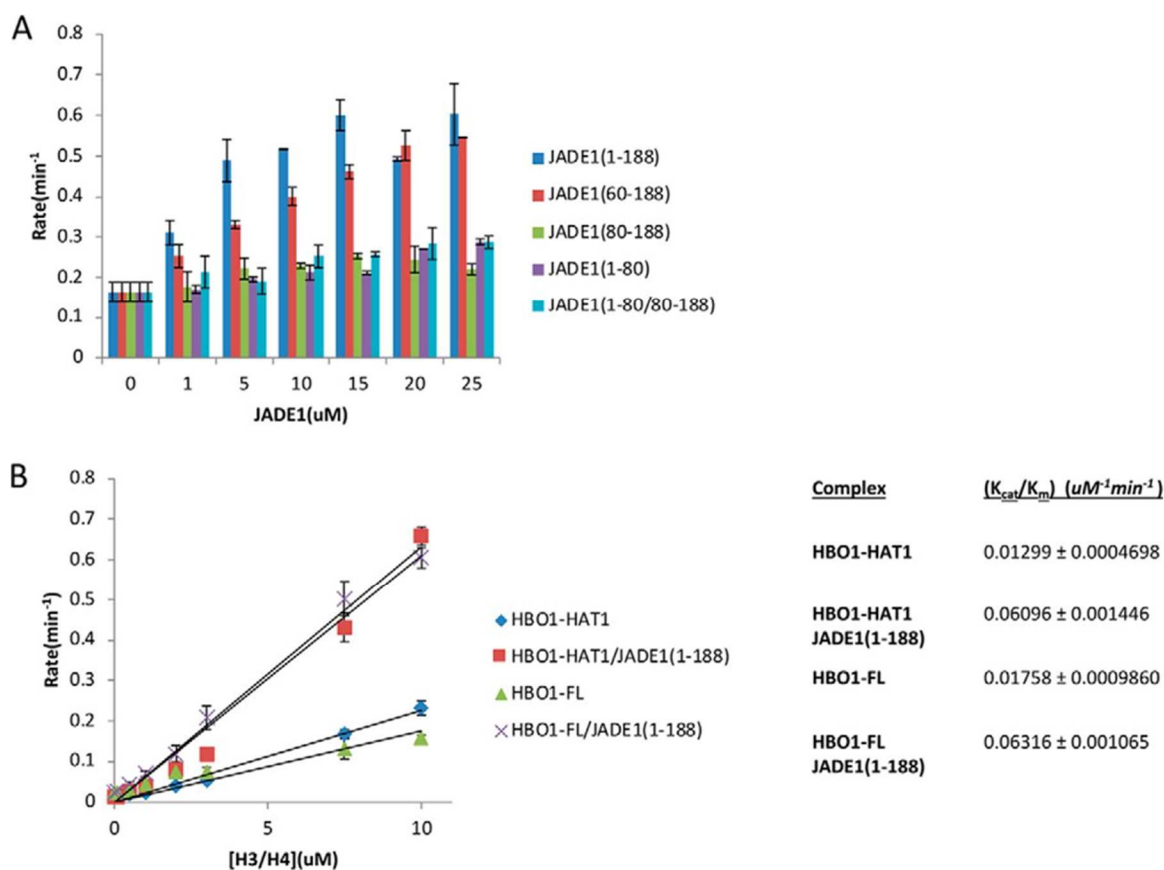


Figure 3. 6JADE1-HHBD and -HCBD work cooperatively to activate HBO1 HAT activity.

(A) titration of JADE1 constructs (1–188, 60–188, 80–188, 1–80, and 1–80/80–188) from 0 to 25 molar excess over the HBO1 HAT domain tested for acetyltransferase activity on the H3/H4 complex. Radioactive counts are converted to enzyme rate as described under “Experimental procedures.” B, plot of the linear range of the Michaelis–Menten plot of HBO1-HAT and HBO1-FL ± JADE1 with H3/H4 as the substrate to calculate the catalytic efficiency (slope, K_{cat}/K_m). Radioactive counts are converted to enzyme rate as described under “Experimental procedures.”

3.3.5 HBO1 contains a region N-terminal to the HAT domain that binds H3/H4 independent of JADE1 binding to H3/H4

Having established that the HHBD and HCBD regions of JADE1 contact H3/H4, we asked if other regions of HBO1 also contribute to histone binding. To address this, we first carried out pull down studies with MBP-HBO1-HAT, JADE1(80-188) and H3/H4 to demonstrate that the HBO1-HAT is unable to directly pull down H3/H4 (**Figure 3.7A**). Although HBO1-HAT does not make direct contact with histones, we asked if the rest of HBO1 has any contribution toward H3/H4 binding independent of JADE1. To address this possibility, we carried out H3/H4 pull down studies with either GST-HBO1-FL or GST alone. This data demonstrated that unlike HBO1-HAT, HBO1-FL is able to pull down H3/H4 indicating that HBO1 also contains an H3/H4 binding site outside of the HAT domain independent of JADE1 (**Figure 3.7B**). Revealing this independent histone binding property of HBO1-FL, we asked if JADE1 and HBO1 can interact with H3/H4 in the presence of each other. To do this, we carried out additional MBP-JADE1 pull-down studies with HBO1-FL and H3/H4 in the presence and absence of JADE1-HHBD. As expected, JADE1(1-188) was able to pull down HBO1-FL independent of H3/H4 binding at HCBD. However, JADE1(108-188) was only able to pull down HBO1-FL in the presence of H3/H4 (**Figure 3.7C**). These data suggest two different findings about HBO1 and H3/H4 interaction. First, although HBO1-HAT only makes JADE1-HHBD dependent contact with H3/H4, HBO1-FL makes JADE1-HHBD independent contact with H3/H4. Second, the fact that H3/H4 does not bind to JADE1-HCBD or HBO1-FL in a mutually exclusive manner suggests that these 2 proteins bind to H3/H4 at different histone binding sites.

To investigate the role of the additional histone binding domain N-terminal to the HBO1-HAT region (HBO1-HBD), we asked if the full length HBO1 shows higher enzymatic efficiency towards the substrate. When compared, the catalytic efficiencies of HBO-HAT and HBO1-FL are comparable to each other. This analysis demonstrates that despite the additional histone binding property of HBO1-FL, HBO1-HBD does not have a significant contribution to the overall HBO1

catalytic efficiency toward H3/H4 and requires JADE1(1-188) to potentiate its acetyltransferase activity (**Figure 3.6B**).

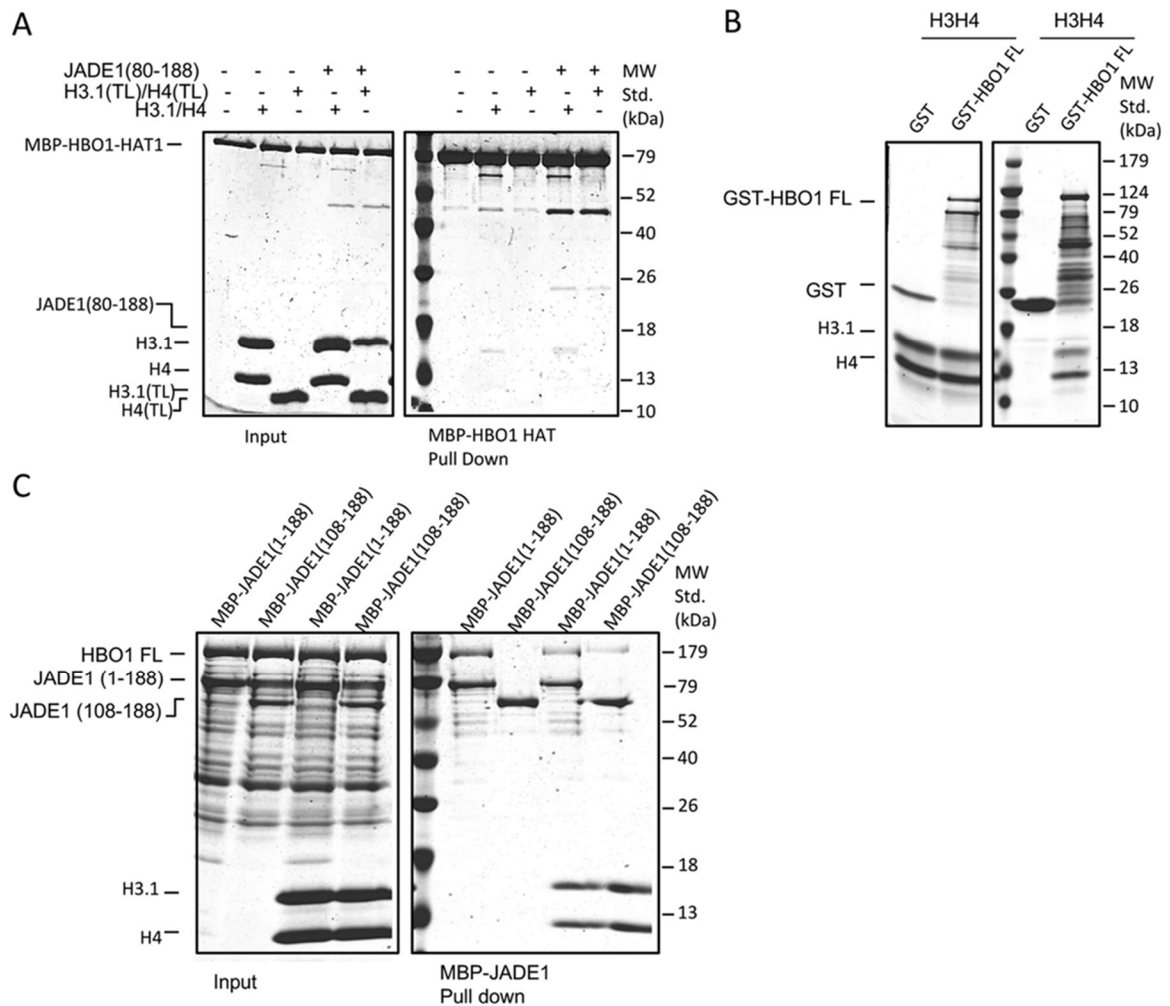


Figure 3. 7HBO1-FL contains an N-terminal H3/H4 binding domain that binds histones independently of JADE1.

(A) MBP-HBO1-HAT pulldown of H3/H4 of H3(TL)/H4(TL) in the presence and absence of JADE1-HCBD, with results resolved on SDS-PAGE. MW Std., molecular weight standard. (B) GST-HBO-FL and GST control pulldown of the H3/H4 complex, with results resolved on SDS-PAGE. Note that JADE1(80–188) and H3.1 co-migrate on SDS-PAGE. (C) MBP-JADE1(1–188) and (108–188) pulldown of HBO1-FL ± H3/H4 complex, with results resolved on SDS-PAGE.

3.3.6 HBO1 may contain an auto-inhibitory domain

We could not detect acetyltransferase activity of full length HBO1 towards H4 peptide (residues 1-19), while the HBO1 HAT domain contained weak but detectable acetyltransferase activity towards the same substrate (**Figures 3.8**). However, when the activity was tested using the radioactive acetylation assay with the intact H3/H4 heterotetramer as the substrate, both full length and the HAT domain of HBO1 showed a similar level of activity (**Figure 3.6B**). In addition, the pull-down analysis shows that although HBO1 does not make contact with the H4 tail, it does contact the histone core at the histone core-binding domain(HBD) located outside of the HAT domain (**Figure 3.4B, 3.7B**). Together, the data demonstrates that full-length HBO1 only shows activity toward H4 tail residues in the presence of the histone core or the JADE scaffold protein, and supports the hypothesis that full-length HBO1 contains an autoinhibitory domain that is relieved upon histone core domain of JADE domain I binding.

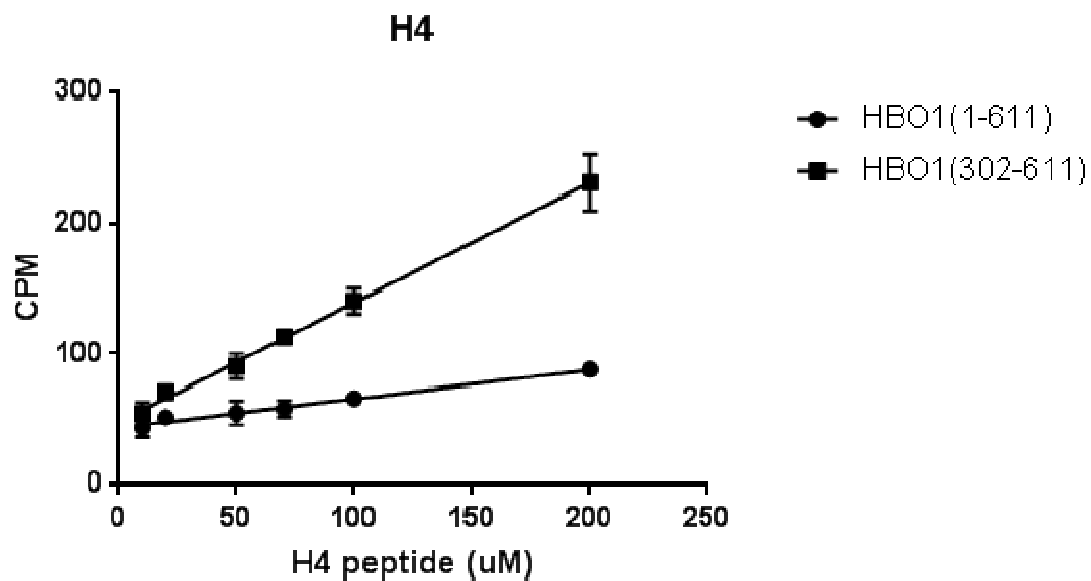


Figure 3. 8Activity comparison of HBO1 full length vs HBO1 HAT domain

Acetyltransferase activity of HBO full length and the isolated HAT domain in response to titration of H4 peptide. The output of the assay is displayed as counts per minute (CPM)

3.3.7 Cellular experiments with JADE1 deletions recapitulate the role of JADE1 in histone and HBO1 interactions and HBO1 acetyltransferase activity

In order to support the conclusions drawn by the *in vitro* interaction studies, we performed co-transfections in HEK 293T cells with vectors expressing different truncation constructs of JADE1 followed by anti-Flag immunoprecipitation and peptide elution of the complexes. Western blot analysis of the purified fractions with progressive N-terminal truncations of JADE1 indicates that deletion of the first 113 amino acids, which includes the HHBD, does not disrupt the interaction of JADE1 with the HBO1-H3/H4 complex in cells (**Figure 3.9A**), while the additional deletion of the JADE1-HCBD abolishes JADE1 interaction with the HBO1-H3/H4 complex, as reported previously (Avvakumov et al., 2012). The observation that JADE1 constructs that contain residues C-terminal to the HHBD (53-842, 114-842 and 199-842) immunoprecipitate with ING4/EAF6 are also consistent with our earlier studies (Avvakumov et al., 2012). We performed a similar experiment using progressive C-terminal deletions of JADE1, demonstrating that the region containing the HHBD is also sufficient to interact with HBO1 in cells (**Figure 3.9B**).

Consistent with the *in vitro* studies, acetylation assays of cell immunoprecipitated JADE1 deletion constructs demonstrates that acetylation activity requires both the HHBD and HCBD regions of JADE1 (**Figure 3.9C**). These results demonstrate that, like observed *in vitro*, while the JADE1 HCBD is required to stabilize the HBO1 complex, the JADE1 HHBD region is required to enable HBO1 acetylation activity towards histones.

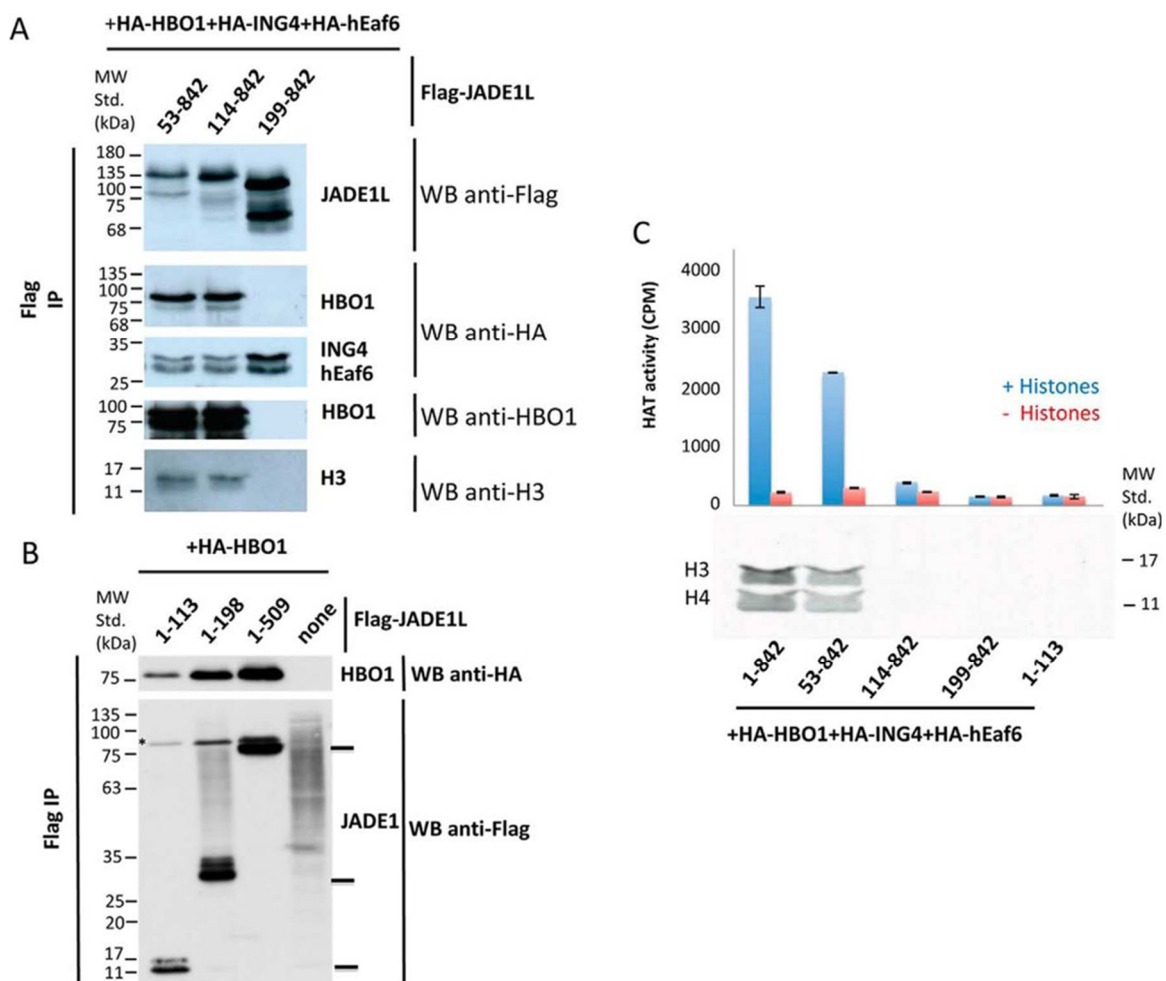


Figure 3. 9JADE1 HHBD and HCBD are both required for HBO1 acetyltransferase activity on histones in cells.

(A) Western blot (WB) analysis of JADE1 complexes with HBO1, H3, ING4, and EAF6 with different N-terminal JADE1 truncations. An anti-HA immunoblot was done first to visualize the HA-tagged subunits of the FLAG-JADE1 complex overexpressed in HEK 293T cells. The

membrane was then reprobed with the HBO1 antibody (showing endogenous HBO1 association beside HA-HBO1) and finally with the anti-FLAG antibody. IP, immunoprecipitation; MW Std., molecular weight standard. (B) HBO1 association with the C-terminal truncated forms of JADE1. Anti-HA was followed by anti-FLAG immunoblot on FLAG-JADE1 complex overexpressed in 293T cells (the asterisk denotes the remaining HBO1 HA signal in the anti-FLAG blot). (C) HAT assay of immunopurified complexes from A. HAT assays were performed on free histones (0.5 µg) with JADE1 complex using the same ratio as for the immunoblotting. Shown is a graphical representation of counts per minute, measured by scintillation, and a fluorogram of the radioactive HAT assay on free histones.

3.4 Discussion

Histone tail acetylation events have been linked to many important cellular processes through controlling chromatin dynamics to mediate various DNA transactions including DNA replication and gene transcription (Strahl and Allis, 2000; Thomas and Voss, 2007). While the structure and catalytic activity of HATs have been well delineated, their mode of substrate-specific acetylation has been less rigorously analyzed. GCN5 has been reported to mediate preferential H3K14 acetylation through making extensive direct contact with the residues of the H3 tail flanking the H3K14 target lysine residue using sequence-based recognition (Rojas et al., 1999). On the other hand, numerous HATs function in multi-subunit complexes where the catalytic efficiency and substrate specificities are determined by subunits outside of the catalytic domain (Lalonde et al., 2013). A recent structural study on the NuA4 HAT complex revealed a sequence/position double recognition mechanism for substrate specificity, whereby the HAT complex binds to the nucleosome through a subunit outside of the catalytic domain to position the H4 tail proximal to the catalytic domain, which then employs sequence-based recognition for sequence-specific acetylation (Xu et al., 2016). Additionally, BRFP2 was recently reported to potentiate and mediate H3 specific acetylation by HBO1 through a short N-terminal fragment that bridges interaction between the HAT domain and H3/H4 (Tao et al., 2017).

In this chapter, we dissected the role of JADE1 in HBO1 HAT activity toward free histones (H3/H4) based on previous studies suggesting that JADE1 plays a key role in HBO1 complex H4 substrate specific acetylation. The in vitro and cellular data presented here demonstrates that robust histone H3/H4 acetylation by the HBO1 requires the HBO1-HAT domain (residues 311-611), in addition to a 21-residue N-terminal JADE1 region (HHBD, residues 60-80), which bridges HBO1-HAT and H3/H4, while a second N-terminal JADE1 region (HCBD, residues 108-188) makes additional H3/H4 contacts within the histone core region (**Figure 3.10A**). The N-terminal region of JADE1 therefore functions as a platform to bring the catalytic HBO1 subunit and the free histone H3/H4 substrate tail together for catalysis. We also demonstrate that HBO1 may contain an auto-inhibitory function, which is dissipated either by binding to the core domain of H3/H4 or JADE. We hypothesize that such an auto-inhibitory domain may serve to insure HBO1 catalytic activity towards cognate histone substrates, over non-histone substrates.

The mechanism of JADE1 activation of the HBO1 HAT complex has similarities and differences to that of BRFP2. Both JADE1 and BRFP2 contain short N-terminal fragments (HHBD in the case of JADE1) that bind to both HBO1 and H3/H4, thus bridging appropriate HBO1-histone interaction for substrate-specific acetylation. Unlike BRFP2, JADE1 contains a second N-terminal histone binding domain (HCBD) that also binds to H3/H4 and cooperates with the HHBD to significantly increase the catalytic efficiency of HBO1 for H4 acetylation.

In addition to the role of the N-terminal region of JADE1 in HBO1 acetylation described here, the role of two C-terminal PHD fingers of JADE1 have previously been characterized. These tandem PHD fingers cooperate to recognize the H3 tail and facilitate targeting of the HBO1 HAT complex to chromatin for nucleosome binding (Foy et al., 2008). Previous studies have suggested that the HBO1 HAT complex is heavily localized to transcriptionally active regions ahead of polymerase to facilitate nucleosome disassembly, consistent with HBO1 complex co-purification with the FACT complex (Saksouk et al., 2009). In the context of transcription within chromatin, JADE1 PHD finger-mediated recognition of methylated histones may facilitate the

subsequent JADE1 mediated HBO1 acetylation of histone H4 to contribute to nucleosome disassembly through increasing chromatin dynamics post nucleosome disassembly and/or provide recognition marks for other proteins involved in transcription elongation. JADE1 has also been linked to HBO1-mediated histone H4 acetylation during DNA replication(Miotto and Struhl, 2010), although the relative roles of the HHBD, HCBD and other C-terminal JADE1 domains in this process remains to be determined.

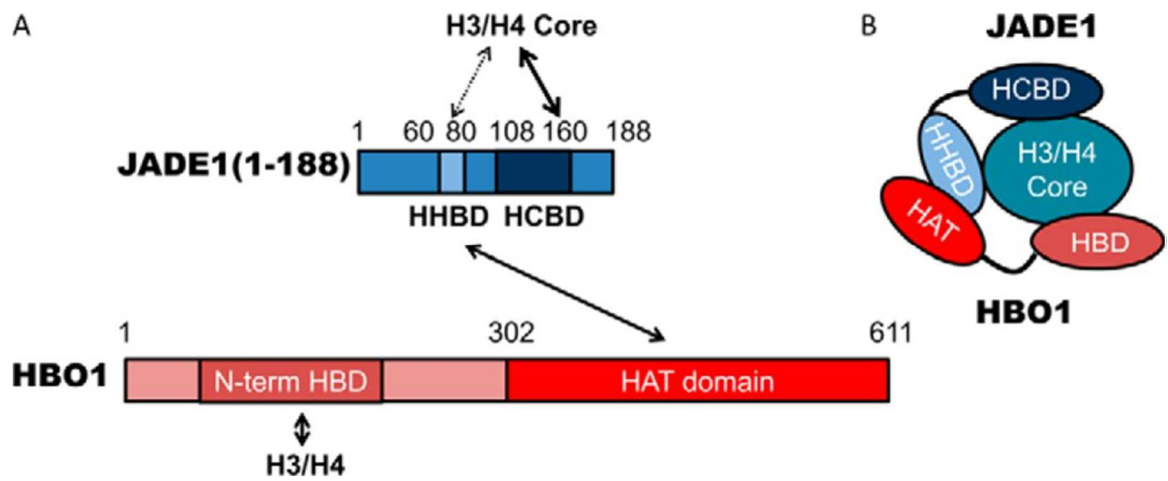


Figure 3. 10 Schematic of the HBO1, JADE1, H3/H4 complex based on the data obtained in this study.

(A) schematic of the JADE1 and HBO1 domains. (B) schematic of JADE1-HBO1 domain interactions.

3.5 Materials and Methods

3.5.1 JADE1/2/3 N-terminal sequence alignment

Sequence alignment for JADE1/2/3 was carried out using ClustalW2(Sievers et al., 2011) and was further formatted using ESPript (3.0) for more optimal sequence homology of JADE homologs(Gouet et al., 2003)

3.5.2 Recombinant HBO1 and JADE1 protein production

HBO1 and JADE1 protein constructs were generated by PCR amplification of the corresponding DNAs, which were cloned into pRSF or pDB.His.MBP vectors using SacI/NotI sites with a cleavable N-terminal affinity tags (GST-,6XHIS-,MBP-). These constructs were grown and expressed using BL21(DE3) cells in LB. Cells were induced for protein overexpression at an OD ~0.6 with 0.5mM IPTG for 16-18 hours at 18 °C. Cells were harvested by centrifugation at 4000 RPM and lysed by sonicating in lysis buffer (20mM Tris(pH 8) 500mM NaCl, 5mM BME, 0.1mg/ml PMSF). The post lysed sample was spun down at 20,000 RPM for 30 min. and the soluble fraction of the post-sonicated sample was applied to either, Pierce Glutathione Agarose resin(Thermo), Amylose resin (NE Biolabs) or Ni-NTA resin (Biosciences) for batch binding at 4°C for 1 hour. Samples were washed with 20 column volumes (CV) of wash buffer (20mM Tris(pH 8), 500mM NaCl, 5mM BME) then eluted with eluant with either 30mM reduced L-glutathione(GST), 20mM maltose monohydrate(MBP), or 300mM Imidazole(6XHIS). Affinity tags were cleaved with TEV protease or were left on for affinity tag pull-downs. Proteins were further purified to homogeneity by either HiTrapQ(GE Healthcare) anion exchange column for JADE1 constructs or HiTrapHP(GE Healthcare) cation exchange for HBO1 constructs. Ion exchange chromatography was performed using a NaCl gradient from 100mM to 1M over 30 CV). To ensure the proper fold of recombinant proteins, they were subjected to size exclusion chromatography(SEC) using a Superdex 200 column in 20mM Tris(pH 8), 500mM NaCl, 5 mM BME. MBP-JADE1(1-188) mutant constructs were generated through site-directed mutagenesis

using Q5 Hot start polymerase(NEB). All of the mutant constructs were verified by DNA sequencing and were expressed and purified using the same protocol mentioned previously for wild-type constructs.

3.5.3 Isothermal Titration Calorimetry

Interaction of HBO1/JADE1 and histone/JADE1 with various JADE1 constructs were quantitatively analyzed using a MicroCal ITC200(Malvern). All proteins were prepared and dialyzed to 20mM HEPES (pH 7.5), 500mM NaCl, 5mM BME before the analysis. All ITC experiments were conducted by injecting 800uM of JADE1 deletion constructs into a calorimetry cell either containing 40uM of HBO1 HAT or H3/H4. JADE1 deletion constructs were injected in 2uL increments every 2 minutes at 20°C. ITC data were analyzed using MicroCal ITC-ORIGIN (Malvern).

3.5.4 Histone and nucleosome core particle production

Each of the histones (H3, H4, H2A, H2B) were expressed and purified as inclusion bodies. Refolding of H2A/H2B, H3/H4 and their purification was performed as previously described(Luger et al., 1999; Ricketts et al., 2015). Tailless constructs H3(36-135) and H4(20-104) were prepared the same way as the wild-type. These refolded histone components, in addition to the 601 nucleosome positioning sequence, were used to form nucleosome core particles as previously described(Luger et al., 1999).

3.5.5 Pull down assay (GST-/MBP-)

2uM of tagged protein with 10uM of untagged protein were incubated together with 50uL of resin for an hour at 4°C. The reactions were carried out in sizing buffer (20mM Tris(pH 8), 500mM NaCl, 5mM BME). Resin was then washed extensively with the same buffer and was collected for analysis on SDS-PAGE gel. All of the pull downs were done side by side with the tag alone under the same conditions as controls for background interactions.

3.5.6 Histone acetyltransferase(HAT) activity assay

HBO1 HAT activity was measured using a previously described HAT assay with ^{14}C labeled Ac-CoA (50-60 mCi/mmol; Moravek)(Yan et al., 2002). A time course for the reaction was initially performed to determine the linear range of enzyme activity at an enzyme concentration of 200nM and saturating concentrations of AcCoA (300uM) and H3/H4 (or nucleosome core particles) (100uM). The HAT reaction was done with 20mM Tris (pH 7.5), 500mM NaCl, 0.25mg/ml BSA for 1 hour at 20°C in a volume of 50uL. The reaction was spotted on P81 filter paper. The positively charged and C^{14} -labeled H4 peptide portion of the histone complex(H3/H4) was captured on the filter paper, while free ^{14}C -labeled Ac-CoA was washed away. The paper was analyzed using Liquid Scintillation(Berndsen and Denu, 2005). The amount of ^{14}C -labeled H4 peptide bound to the paper is directly proportional to the amount of acetyl group transferred from the cofactor to peptide, thus the data was used to evaluate HBO1 acetyltransferase activity(Berndsen and Denu, 2005). All reactions were done in duplicate. Using the known concentration series of Ac-CoA, a standard curve was generated. The standard curve was used to convert radioactive counts to molar units.

3.5.7 JADE1-HBO1 complexes cotransfection in 293T cells

JADE1L, HBO1, hEAF6 and ING4 constructs were generated by PCR amplification of the corresponding DNAs, which were cloned into pcDNA3 vector encoding a N-terminal Flag tag using BglII/Xho1 sites or a HA tag. These plasmids were then used to cotransfected 293T cells by the calcium phosphate method for protein complex overexpression with HA-HBO1/HA-ING4/HA-hEAF6 and full length or N-terminal truncated 3xFlag-JADE1L: amino acid 1-842, 53 -842, 114-842, 199-842 and 1-113. A second set of cotransfection was done using C-terminal truncated 1x Flag JADE1: the first 113, 198 or 509 amino acids were overexpressed in combination with HA-HBO1. Cells were harvested 48h post-cotransfection and lysed in high salt buffer (450mM NaCl, 50mM Tris-HCl pH 8.0, 1% TX-100, 2mM MgCl_2 , 0.1mM ZnCl_2 , 2mM EDTA, 10% glycerol)

supplemented with protease inhibitor mixture. The NaCl concentration was reduced to 225mM and the whole cell extract centrifuged at 14,000 RPM for 30 minutes. The Flag JADE1 was purified from the soluble fraction using anti-Flag M2 affinity beads (Sigma) for 2h at 4°C on rotating wheel. Flag beads were next washed with the 225mM NaCl buffer and eluted with 400ug/ml of 3X or 1X Flag peptide (Sigma) for the N-terminal or the C-terminal truncations, respectively.

3.5.8 Immunoblotting

The amount of the different JADE1 construct was normalized empirically following SDS-PAGE and transfer onto nitrocellulose membrane. Anti-Flag M2 conjugated to horse radish peroxidase (HRP; Sigma) was used at a 1:10,000 dilution and the immunoblots were visualized using a Western Lightning plus-ECL reagent (Perkin-Elmer). Anti-HA 3F10 (Roche, rat polyclonal) and anti-HBO1 (Novus, rabbit polyclonal) antibodies were used at a 1:1,000 dilution. HRP conjugated secondary anti-rat and anti-rabbit antibodies (Sigma) were used at a 1:10,000 dilution.

3.5.9 HAT activity assays on purified JADE1 complexes

HBO1 activity in the purified JADE1 complexes was measured with 0.125uCi of ³H labeled Ac-CoA (2.1Ci/mmol; PerkinElmer Life Sciences). The amount of the different purified JADE1 complexes used in the HAT assays were normalized based on the immunoblotting data. The HAT reactions were performed in a volume of 15 or 20ul using 0.5ug of human free histones or native short oligonucleosomes as substrate, respectively, in HAT buffer (50mM Tris-HCl (pH 8), 50mM KCl, 10mM sodium butyrate, 5% glycerol, 0.1mM EDTA, 1mM dithiothreitol) for 1 hour at 30°C. The reactions were then captured on P81 filter paper, the free ³H-labeled Ac-CoA was washed away and the paper was analyzed using Liquid Scintillation. The ³H counts were used to evaluate HBO1 acetyltransferase activity in the complexes containing different JADE1 constructs.

Chapter 4 -Conclusion and Future direction

4.1 Overview

In this thesis, we identified and characterized the importance of the scaffold protein JADE in HBO1 HAT activity using biochemical and biophysical analysis. In this chapter we discuss our findings and possible future directions to further understand the function of the scaffold protein JADE in the acetyltransferase activity of HBO1.

4.2 JADE as the scaffold protein for the HBO1 HAT complex

Prior to our study, it was known that JADE functions as a platform for other subunits in the HBO1 HAT complex. However, the actual role of JADE in the acetyltransferase activity of HBO1 was unknown. In this thesis, using a various biochemical and biophysical techniques such as affinity tag pull-down, radioactive acetylation assay, and ITC we have demonstrated that the function of an N-terminal region of JADE is to recruit the H3/H4 histone substrate within close proximity to HBO1 for acetylation. Although our study did not address any potential allosteric effects of JADE on the HAT domain of HBO1, it remains a distant possibility and further study is necessary to address this possibility.

Recent crystallographic information for the *yeast* NuA4 HAT Complex reveals that the Epl1 scaffold protein contains a loop that is responsible for both binding to the HAT domain of Esa1 and the nucleosome. The loop functions in two ways, hence the name, dual functional loop(DFL). First, binding of the DFL to the HAT domain of Esa1 rearranges the active site into a conformation more suitable for catalysis. Second, adjacent to the HAT binding region in the DFL is the nucleosome-binding domain, which helps position the NuA4 complex proximal to the histone H4 tail substrate(Xu et al., 2016). However, a recent crystal structure of the HBO1 HAT domain in complex with a fragment of BRPF2 reveals that BRPF2 positions the substrate proximal to the active site without allosterically modulating the active site as observed for the Esa1/Epl1 complex(Tao et al., 2017). Whether the JADE functions in a manner more akin to Epl1 or BRPF2 remains unclear and requires further investigation.

4.3 Kinetic approach to understanding the possible allosteric affects of JADE1 on HBO1 HAT domain

In our study, using MBP pull-down and Isothermal titration calorimetry (ITC), we demonstrated that 2 regions of JADE1(HHBD,HCBD) bind to the core of H3/H4 with different affinities. In combination with our biochemical data, we hypothesize that the HCBD of JADE1 anchors the HBO1 HAT complex on the histones and the HHBD orients the HAT domain by acting as a bridge between the HAT domain and the substrate. However, it remains unclear if the JADE-HHBD has any sort of allosteric effects on the HBO1 catalytic site. Although more extensive study is necessary to fully confirm, we have some data suggesting a possible allosteric affect of JADE1 on HBO1. Our biochemical studies in Chapter 3 reveals that both JADE and HBO1 bind to the core of H3/H4 and neither have additional contact with the H4 tail. Using this information, we proposed a model where JADE1 acts as a scaffold to mediate the interaction between the active site and core of the substrate, H3/H4. Our proposed model suggests that the interaction between JADE and the H3/H4 core locates the H4 tail proximal to the active site of HBO1, increasing the local concentration of the H4 tail. If this is the only way JADE activates HBO1, we should not observe JADE stimulating HBO1 activity when the H4(1-19) peptide is used as substrate. However, the fact that we saw stimulation of HBO1 activity towards the H4 tail peptide(1-19) in the presence of JADE1 leads us to think that there might be more than just recruitment of the substrate by binding at the histone core (**Figure 2.10**).

Although a structure of the HBO1/JADE complex would help fully elucidate any potential allosteric contributions of JADE, we can learn whether JADE1 has allosteric effects on the HAT domain through carrying out a series of biochemical assays. To better understand the effect of the JADE HHBD on the HBO1 HAT domain, we can conduct the previously described radioactive acetylation assay using various combinations of the histone core and tail as the substrate. Using the HBO1/JADE1 complex we can test the acetyltransferase activity towards various histone combinations as the substrate; H3/H4, (TL)H3/(TL)H4/H4 tail, H4 tail. The comparison of the

kinetic parameters of the HBO1 HAT complex towards these substrates would be a useful tool to fully understand how JADE1 activates HBO1 for its acetyltransferase activity.

4.4 H3/H4 core chemical acetylation

Using H3/H4 as the substrate we determined the enzymatic rate of different HBO1 HAT complexes by calculating the initial slope of the Michaelis-Menten model. When we performed the acetylation assay to understand the kinetics of the HBO1 HAT complex using H3/H4 as the substrate, we were not able to carry out the full Michaelis-Menten kinetics due to high levels of chemical acetylation on the core of the histones. Further exploration is necessary to develop an assay with diminished chemical acetylation. The ability to screen activity of the different HAT complexes in high concentration of histones will allow us to fully determine the Michaelis-Menten enzyme kinetics and characterize different histone acetyltransferases complexes.

4.5 Purification of the HBO-HAT holo complex.

Co-infection of the 4 viruses containing FLAG-HBO1, HA-JADE3, HA-Eaf6, and HA-ING4 in Sf9 cells leads to a stable expression of each subunit and formation of a 4-protein complex that could be purified in solution. However, the final yield of this HAT complex in the was low (~1mgs/ml). We speculate that these human proteins are very complicated in their biology and put a lot of stress on our Sf9 expression system as we co-infect 4 different viruses into the same cell. We need to develop better methods to express and co-purify this 4-protein HAT complex for further studies. Recently Peters and co-workers developed a method to assemble up to 25 genes in one expression vector, leading to stable expression and complex formation(Weissmann et al., 2016). We can utilize this system to clone 4 of these HBO1 HAT complex genes into one expression vector, which may lead to a more stable complex with higher product yield. The ability to purify this complex in a high amount will help us to further characterize the activity and structure of this complex.

4.6 HBO1 inhibitor development

Because of the link between HBO1 and other MYST HAT proteins to cancer, and particularly with chromosomal translocations, there has been a significant interest in developing protein-selective MYST inhibitors (Suzuki and Miyata, 2006; Zheng et al., 2008), but with very limited success. Zheng and coworkers have developed hTIP60 inhibitors that are competitive with acetyl-CoA (A-CoA) with IC_{50} values ranging from 149 μ M to 400 μ M. These inhibitors bind to the A-CoA binding pocket of the HAT domain in a manner that is competitive with A-CoA. Although some inhibitors show better IC_{50} than the known natural product inhibitor curcumin (diferuloylmethane), they do not show selectivity amongst different HATs (Wu et al., 2011). In addition, Zheng and coworkers prepared analogs of the known natural product HAT inhibitor, anacardic acid, resulting in the identification of a series of 6-alkylsalicylates with selectivity for MYST proteins over Gcn5 and p300, with the most potent compound competing against A-CoA, with IC_{50} values of 74 μ M and 47 μ M for hTIP60 and hMOF, respectively (Ghizzoni et al., 2012). Their docking study shows that the most potent inhibitor binds either at the core (A-CoA binding pocket) or around the MYST domain. Due to the structural homology and similar catalytic mechanism between different MYST acetyltransferases, it is not a surprise that the IC_{50} against different MYST enzymes is comparable. The MYST family HATs function as members of multi-protein complexes, as is true for many other acetyltransferases (Avvakumov et al., 2012). The various associated subunits of the MYST HAT complex are especially important in catalytic activities and substrate specificities of these HAT complexes. (Shahbazian and Grunstein, 2007) This phenomenon opens the possibility of targeting other subunits of the respective MYST HAT complexes or their interactions with the catalytic subunit to develop MYST selective inhibitors. In the case of HBO1, targeting JADE or its interaction with HBO1 could be a fruitful avenue of investigation.

Appendix A -Biochemical studies on the Nat10 acetyltransferase

A.1 Abstract

NAT10 has been shown to be a cancer biomarker and the first line of response to genotoxins. Recently, the inhibition of NAT10 was shown to alleviate LNMA depleted laminophetic cells. Using this information, we carried out biochemical studies to understand the function and inhibition of NAT10.

A.2 Introduction

LMNA, which encodes lamin A and C, has an important function in contributing to the shape of nuclear lamina and overall organization of chromatin. Mutations in these genes cause laminopathies, including the accelerated-aging disease Hutchinson-Gilford progeria syndrome (HGPS) (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003; Worman and Bonne, 2007). Proper regulation of lamin A expression is important, as any misregulation of expression or localization can lead to various cancers (Broers et al., 1993; Galiova et al., 2008; Moss et al., 1999). As the depletion of LMNA causes defects to the shape of the nuclear lamina leading to global chromatin relaxation, it was thought that acetylation or deacetylation may play an important role in improving the nuclear shape through modulating chromatin architecture. For this reason, Jackson and coworkers included a library of HAT and HDAC inhibitors in screening for small molecules that alleviated the defects in nuclear shape of the LMNA depleted cells. Using this method they identified a HAT inhibitor 4-(4-chlorophenyl)-2-(2-cyclopentylidenehydrazinyl). This molecule showed a restoration of nuclear circularity and global chromatin compaction in siLMNA cells lacking Lamin A/C. This compound completely rescued nuclear-shaping within 12 hours of treatment. The fact that the molecule was directly involved in the remodeling the nuclear lamina resulted in the name Remodelin for this newly identified compound. Using click-chemistry Jackson and coworkers identified the target for Remodelin as NAT10(Larrieu et al., 2014).

NAT10 has been previously linked with SUN1 nuclear envelope protein(Chi et al., 2007). NAT10 has been shown to be co-localized primarily to the cell nucleolus(Liu et al., 2007; Shen et al., 2009) contributing to overall maintenance of the nuclear shape(Amin et al., 2007; Amin et al., 2008). In addition, NAT10 expression level increases as a response to genotoxic agents(Liu et al., 2007). NAT10 was also shown to be a biomarker for cancer as the localization of NAT10 is highly correlated with tumor grading(Shen et al., 2009). Using tubulin as a substrate, they showed that NAT10 harbors acetyltransferase activity towards tubulin and Remodelin was able to show the same level of inhibition as a catalytic mutant. NAT10 inhibition in laminopathic cells is suggested to reduce microtubule anchorage, which then releases the external force of the nuclear envelope, leading to reshaping of the nucleus and improvement in overall health of the cell(Larrieu et al., 2014). In a recent study, NAT10 was shown to be involved in ribosome biogenesis and rRNA acylation. In this study, Lafontaine and coworkers proposed a new mechanism that NAT10 inactivation leading to correction of laminopathic cells may be due to reduced ribosome biogenesis(Sharma et al., 2015).

Although previous studies have identified a small molecule inhibitor that can alleviate laminopathic cells, the molecular basis of how Remodelin inhibits the acetyltransferase activity of NAT10 is still unknown. Having this gap in knowledge, we planned to investigate the general function of NAT10 and use X-crystallography to understand the molecular basis for function and inhibition of NAT10. We could then use this knowledge to potentially develop more potent inhibitor for NAT10 specific diseases.

NAT10 is a very large protein that contains various additional domains such as DUF1725, ATPase, RNA helicase, and a tRNA binding domain in addition to the acetyltransferase domain(Larrieu et al., 2014) (**Figure A.1A**). Other domains of NAT10 have functions outside of acetylation such as substrate recognition and RNA binding(Sharma et al., 2015).

A.3 Result

In order to biochemically characterize NAT10, we set out to prepare recombinant protein. We initially attempted to make the full length NAT10 protein with very limited success. Both Baculovirus sf9 and *E.coli* expression systems were not able to produce soluble full length NAT10. We then tried to make various recombinant constructs of the NAT10 HAT domain using evolutionary conservation of the acetyltransferase domain to guide construct design (**Figure A.1B**). From our construct screening of the HAT domain, we identified a construct of NAT10 (494-753) that led to good expression and soluble purification (**Figure A.2**). The isolated acetyltransferase domain of NAT10 showed a proper fold.

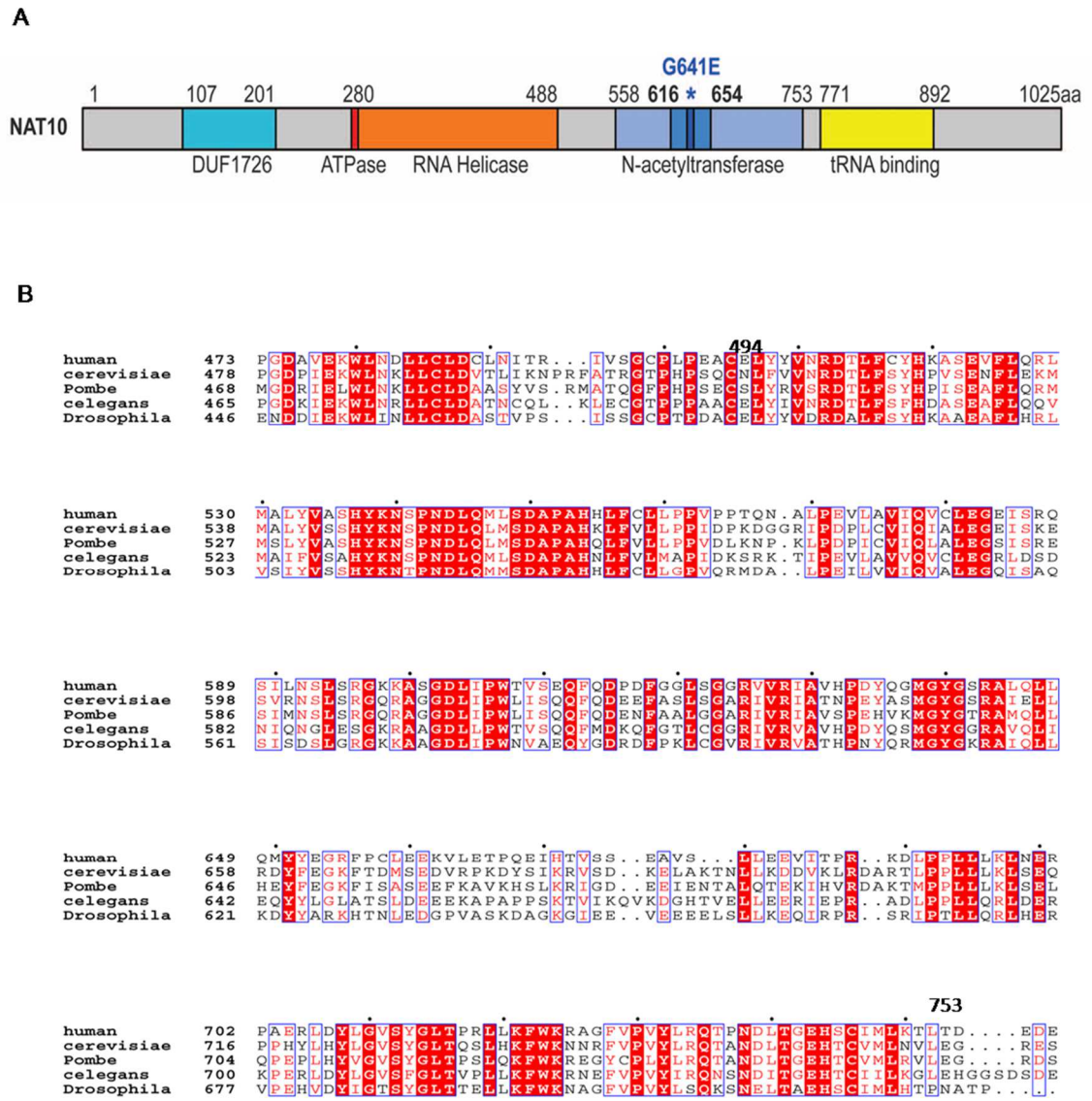


Figure A. 1 NAT10

(A) Schematic representation of NAT10 (B) Sequence alignment of the acetyltransferase domain of different orthologs of NAT10. Larrieu et al, (2014) Science

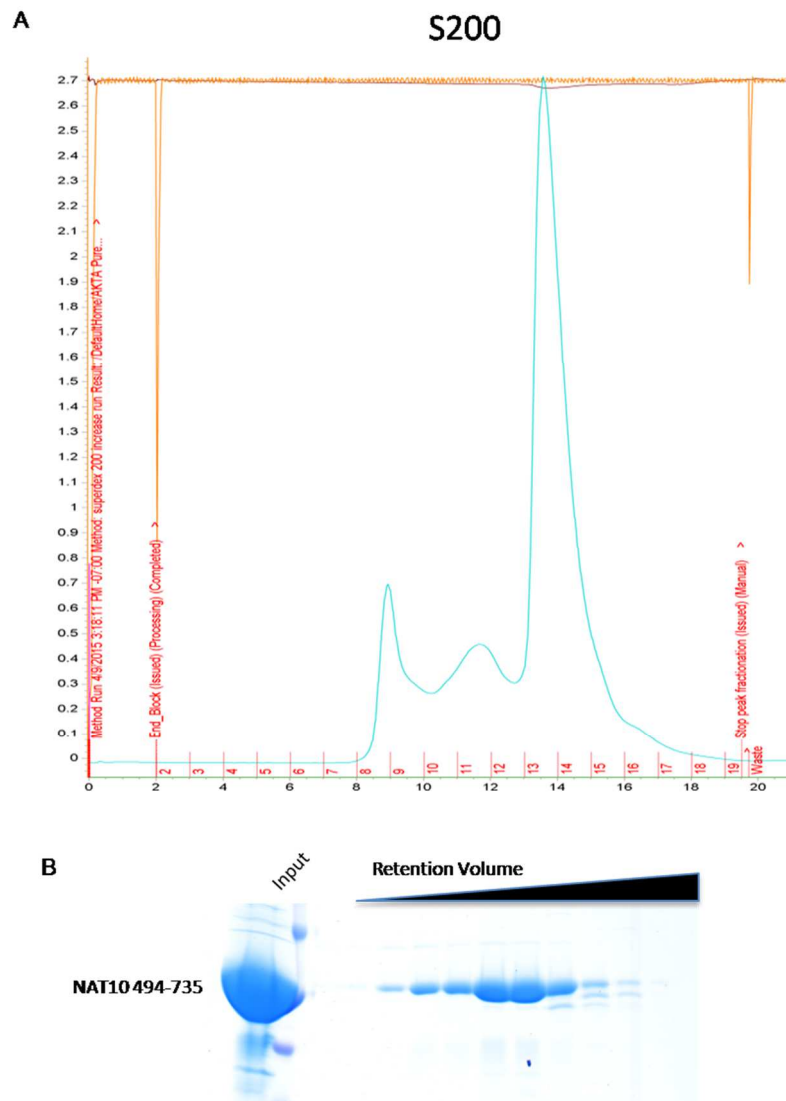


Figure A. 2 Purification of NAT10 acetyltransferase domain

(A) S200 size exclusion chromatography run of the isolated acetyltransferase of NAT10 (494-735)

(B) S200 run resolved on SDS-PAGE gel.

Using this NAT10 fragment, we performed Isothermal titration calorimetry (ITC) experiments with NAT10 (494-735) and either Acetyl-CoA, Acetonyl-CoA, or CoA. The ITC data show that NAT10 (494-753) is able to bind to acetyl-CoA, Acetonyl-CoA, and CoA with K_d values of 5.26uM, 7.04uM, 13.45uM, respectively (Figure Appendix A.3). The fact that acetyl-CoA and the derivatives stably bound to the HAT domain suggested that the acetyltransferase domain was properly folded. The previous study by Jackson et al, using a 3D model suggested that Remodelin may bind to the A-CoA binding pocket of the HAT domain. Taking this information, we also performed ITC with NAT10 (494-753) and Remodelin (Synthesized by Dr. Jeffrey Winkler's laboratory, Department of Chemistry, University of Pennsylvania). From this experiment, we were not able to detect binding of Remodelin to NAT10 (Figure Appendix A.3.3).

We performed Sypro Orange Thermal Shift assays with NAT10 (494-735) alone and in the presence of A-CoA and Remodelin (Figure Appendix A.3.4). The apparent melting temperature of the apo HAT domain was $\sim 42.3^\circ\text{C}$ and the apparent melting temperature of the acetyltransferase domain in the presence of acetyl-CoA increased by $\sim 8^\circ\text{C}$. However, in correlation with the ITC data, Remodelin did not further stabilize the acetyltransferase domain of NAT10. Together, this data suggested that Remodelin does not directly bind the acetyltransferase domain of NAT10 (Figure A.4).

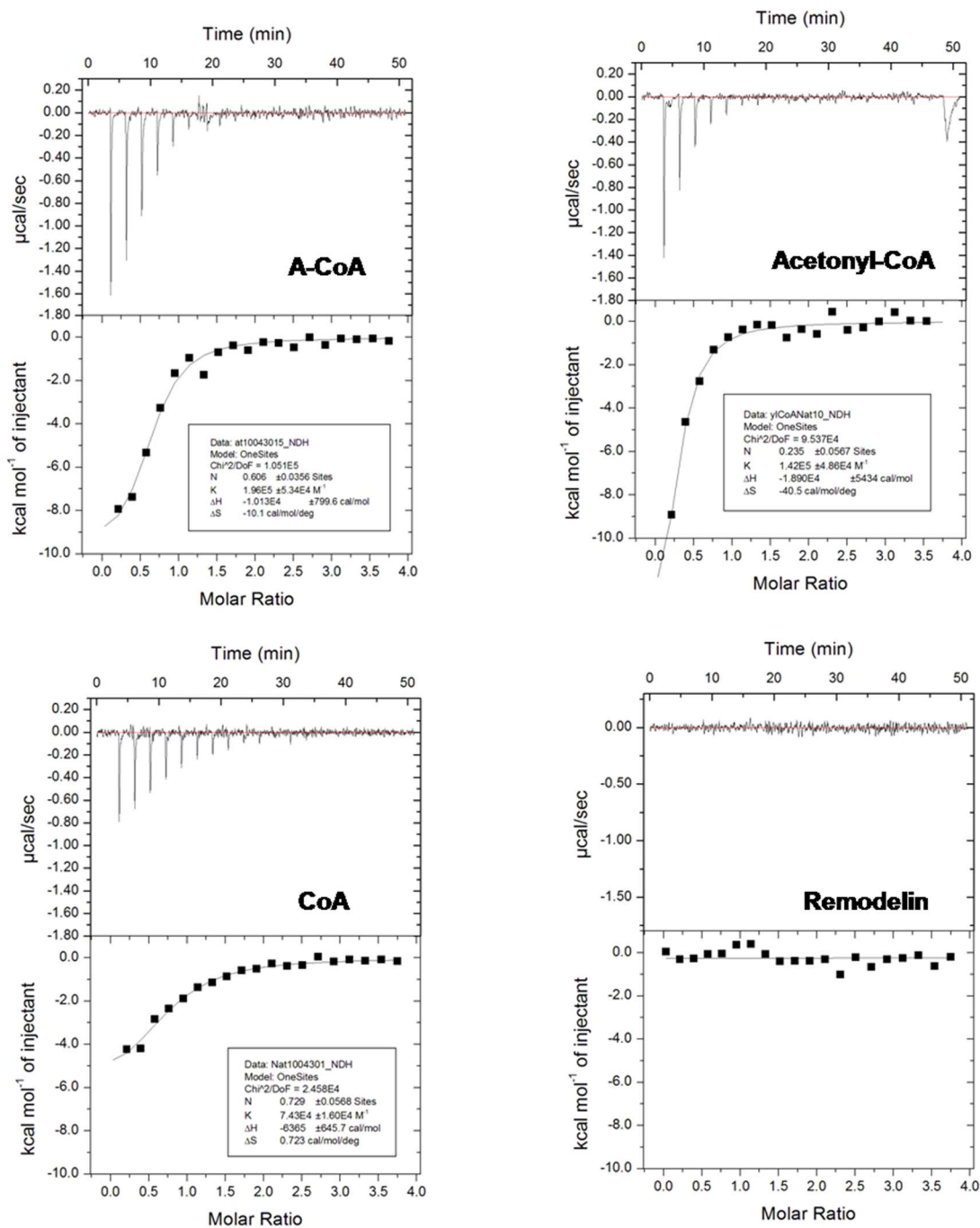


Figure A. 3 ITC of NAT10(494-753) with A-CoA, Acetonyl-CoA, CoA, Remodelin

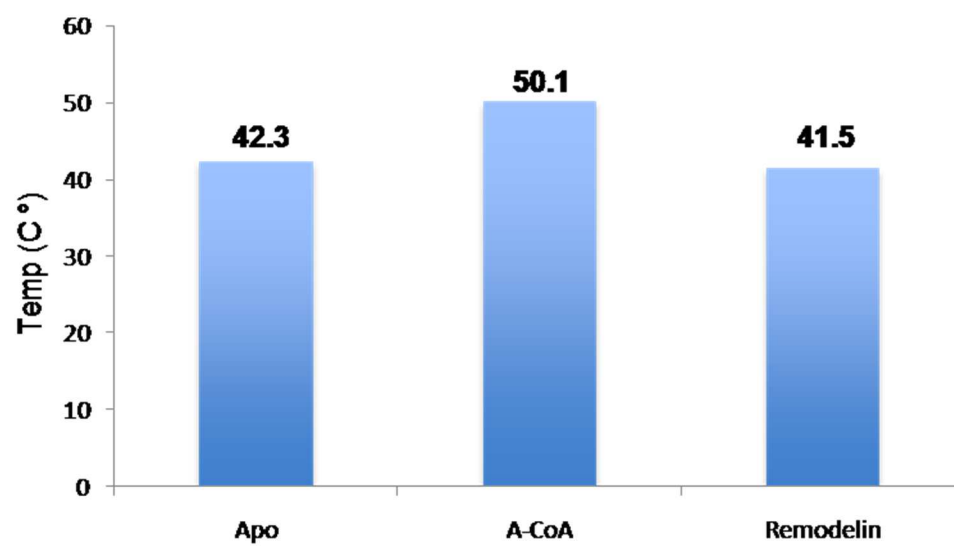


Figure A. 4 Thermal Shift Assay of NAT10 (494-753) with A-CoA, Remodelin

After demonstrating that NAT10 (494-753) binds tightly to acetyl-CoA but not to Remodelin, we tested the ability of the NAT10 acetyltransferase domain to modify the proposed α -tubulin substrate (Shen et al., 2009). We tested the acetyltransferase activity of the NAT10 HAT domain against 3 different substrates; microtubules, MAP-enriched tubulin, and α -tubulin. We monitored NAT10 acetylation using two methods: the previously described radioactivity assay, as well as western blot with an anti-pan-acetyl antibody to detect the activity of NAT10 at two different reaction temperatures (**Figure A.5,6**). However, we were not able to detect activity of NAT10 with either assays.

A.4 Discussion

Although we were not able to confirm the role of Remodelin in the inhibition of NAT10 activity towards tubulin or microtubules, RNA has more recently been identified as the substrate for NAT10 (Ito et al., 2014). A recent publication indicated that cytidine acetylation by NAT10 was shown to be critical in ribosome biogenesis. For future studies we plan to investigate the role of NAT10 in RNA acetylation.

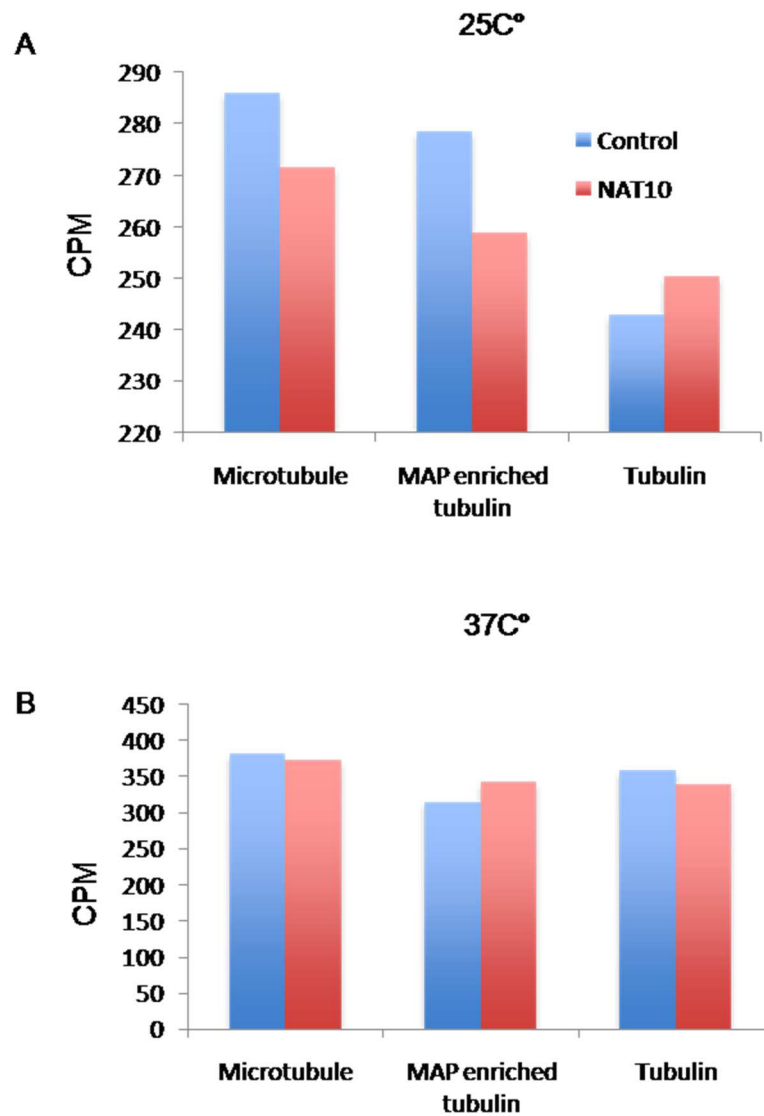


Figure A. 5 Radioactive assay of NAT10 acetyltransferase activity.

Acetyltransferase activity was measured using Microtubule, MAP enriched tubulin, Tubulin as substrates. The output of the assay is displayed as counts per minute (CPM) (A) 25C°(B) 37C°

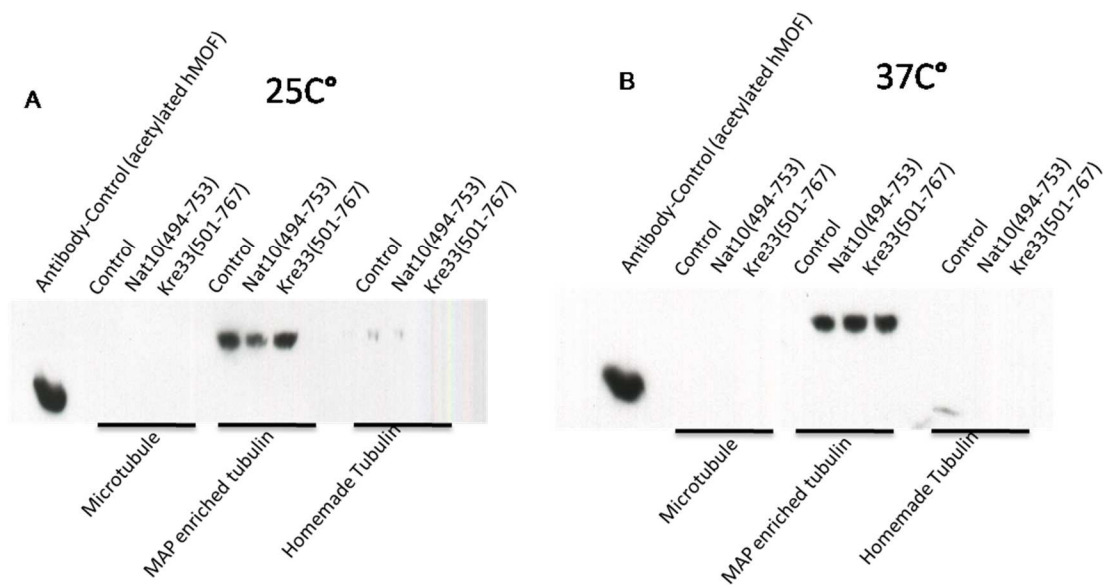


Figure A.6 Radioactive assay of NAT10 acetyltransferase activity.

Acetyltransferase activity was measured using Microtubule, MAP enriched tubulin, Tubulin as substrates. The output of the assay was detected using anti-acetyl-lysine antibody. (A) 25C° (B) 37C°

A.5 Materials and Methods

A.5.1 NAT10 orthologs sequence alignment

Sequence alignment for NAT10 from different organisms was carried out using ClustalW2(Sievers et al., 2011) and was further formatted using ESPript (3.0) for more optimal sequence homology of NAT10 orthologs(Gouet et al., 2003)

A.5.2 Recombinant NAT10 protein production

NAT10 protein constructs were generated by PCR amplification of the corresponding DNAs which were cloned into pDB.His.MBP vectors using SacI/NotI sites with a cleavable N-terminal affinity tags (MBP-). These constructs were grown and expressed using BL21(DE3) cells in LB. Cells were induced for protein overexpression at an OD ~0.6 with 0.5mM IPTG for 16-18 hours at 18 °C. Cells were harvested by centrifugation at 4000 RPM and lysed by sonicating in lysis buffer (20mM Tris(pH 8) 500mM NaCl, 5mM BME, 0.1mg/ml PMSF). The post lysed sample was spun down at 20,000 RPM for 30 min. and the soluble fraction of the post-sonicated sample was applied to Amylose resin (NE Biolabs) for batch binding at 4°C for 1 hour. Samples were washed with 20 column volumes (CV) of wash buffer (20mM Tris(pH 8), 500mM NaCl, 5mM BME) then eluted with eluant with 20mM maltose monohydrate(MBP). Affinity tag was cleaved with TEV protease. To ensure the proper fold of recombinant proteins, they were subjected to size exclusion chromatography(SEC) using a Superdex 200 column in 20mM Tris(pH 8), 500mM NaCl, 5 mM BME.

A.5.3 Tubulin Purification

Tubulin was purified from porcine brain according to Gell *et al*(Gell et al., 2011). Tubulin was assembled into microtubule by methods described (Piperno et al., 1987)

A.5.4 NAT10 Acetylation assays

Acetylation assay on microtubule, tubulin, MAP-enriched tubulin was done as described in the methods(Friedmann et al., 2012).

Reference

- Allfrey, V.G., and Mirsky, A.E. (1964). Structural Modifications of Histones and their Possible Role in the Regulation of RNA Synthesis. *Science* *144*, 559.
- Allis, C.D., Berger, S.L., Cote, J., Dent, S., Jenuwien, T., Kouzarides, T., Pillus, L., Reinberg, D., Shi, Y., Shiekhata, R., *et al.* (2007). New nomenclature for chromatin-modifying enzymes. *Cell* *131*, 633-636.
- Amin, M.A., Matsunaga, S., Ma, N., Takata, H., Yokoyama, M., Uchiyama, S., and Fukui, K. (2007). Fibrillarin, a nucleolar protein, is required for normal nuclear morphology and cellular growth in HeLa cells. *Biochem Biophys Res Commun* *360*, 320-326.
- Amin, M.A., Matsunaga, S., Uchiyama, S., and Fukui, K. (2008). Depletion of nucleophosmin leads to distortion of nucleolar and nuclear structures in HeLa cells. *Biochem J* *415*, 345-351.
- Avvakumov, N., Lalonde, M.E., Saksouk, N., Paquet, E., Glass, K.C., Landry, A.J., Doyon, Y., Cayrou, C., Robitaille, G.A., Richard, D.E., *et al.* (2012). Conserved molecular interactions within the HBO1 acetyltransferase complexes regulate cell proliferation. *Mol Cell Biol* *32*, 689-703.
- Berndsen, C.E., and Denu, J.M. (2005). Assays for mechanistic investigations of protein/histone acetyltransferases. *Methods* *36*, 321-331.
- Berndsen, C.E., and Denu, J.M. (2008). Catalysis and substrate selection by histone/protein lysine acetyltransferases. *Curr Opin Struct Biol* *18*, 682-689.
- Broers, J.L., Raymond, Y., Rot, M.K., Kuipers, H., Wagenaar, S.S., and Ramaekers, F.C. (1993). Nuclear A-type lamins are differentially expressed in human lung cancer subtypes. *The American journal of pathology* *143*, 211-220.
- Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y., and Allis, C.D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* *84*, 843-851.
- Chi, Y.H., Haller, K., Peloponese, J.M., Jr., and Jeang, K.T. (2007). Histone acetyltransferase hALP and nuclear membrane protein hsSUN1 function in decondensation of mitotic chromosomes. *The Journal of biological chemistry* *282*, 27447-27458.
- De Sandre-Giovannoli, A., Bernard, R., Cau, P., Navarro, C., Amiel, J., Boccaccio, I., Lyonnet, S., Stewart, C.L., Munnich, A., Le Merrer, M., *et al.* (2003). Lamin A truncation in Hutchinson-Gilford progeria. *Science* *300*, 2055.
- Doyon, Y., Cayrou, C., Ullah, M., Landry, A.J., Cote, V., Selleck, W., Lane, W.S., Tan, S., Yang, X.J., and Cote, J. (2006). ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Molecular cell* *21*, 51-64.
- Drazic, A., Myklebust, L.M., Ree, R., and Arnesen, T. (2016). The world of protein acetylation. *Biochim Biophys Acta* *1864*, 1372-1401.
- Drazic, A., and Winter, J. (2014). The physiological role of reversible methionine oxidation. *Biochim Biophys Acta* *1844*, 1367-1382.

Duong, M.T., Akli, S., Macalou, S., Biernacka, A., Debeb, B.G., Yi, M., Hunt, K.K., and Keyomarsi, K. (2013). Hbo1 is a cyclin E/CDK2 substrate that enriches breast cancer stem-like cells. *Cancer research* 73, 5556-5568.

Eriksson, M., Brown, W.T., Gordon, L.B., Glynn, M.W., Singer, J., Scott, L., Erdos, M.R., Robbins, C.M., Moses, T.Y., Berglund, P., *et al.* (2003). Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature* 423, 293-298.

Feng, Y., Vlassis, A., Roques, C., Lalonde, M.E., Gonzalez-Aguilera, C., Lambert, J.P., Lee, S.B., Zhao, X., Alabert, C., Johansen, J.V., *et al.* (2016). BRPF3-HBO1 regulates replication origin activation and histone H3K14 acetylation. *The EMBO journal* 35, 176-192.

Foy, R.L., Song, I.Y., Chitalia, V.C., Cohen, H.T., Saksouk, N., Cayrou, C., Vaziri, C., Cote, J., and Panchenko, M.V. (2008). Role of Jade-1 in the histone acetyltransferase (HAT) HBO1 complex. *The Journal of biological chemistry* 283, 28817-28826.

Friedmann, D.R., Aguilar, A., Fan, J., Nachury, M.V., and Marmorstein, R. (2012). Structure of the alpha-tubulin acetyltransferase, alphaTAT1, and implications for tubulin-specific acetylation. *Proc Natl Acad Sci U S A* 109, 19655-19660.

Galiova, G., Bartova, E., Raska, I., Krejci, J., and Kozubek, S. (2008). Chromatin changes induced by lamin A/C deficiency and the histone deacetylase inhibitor trichostatin A. *Eur J Cell Biol* 87, 291-303.

Gell, C., Friel, C.T., Borgonovo, B., Drechsel, D.N., Hyman, A.A., and Howard, J. (2011). Purification of tubulin from porcine brain. *Methods Mol Biol* 777, 15-28.

Ghizzoni, M., Wu, J., Gao, T., Haisma, H.J., Dekker, F.J., and George Zheng, Y. (2012). 6-alkylsalicylates are selective Tip60 inhibitors and target the acetyl-CoA binding site. *Eur J Med Chem* 47, 337-344.

Gouet, P., Robert, X., and Courcelle, E. (2003). ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. *Nucleic acids research* 31, 3320-3323.

Haberland, M., Johnson, A., Mokalled, M.H., Montgomery, R.L., and Olson, E.N. (2009a). Genetic dissection of histone deacetylase requirement in tumor cells. *Proc Natl Acad Sci U S A* 106, 7751-7755.

Haberland, M., Montgomery, R.L., and Olson, E.N. (2009b). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet* 10, 32-42.

Havasi, A., Haegele, J.A., Gall, J.M., Blackmon, S., Ichimura, T., Bonegio, R.G., and Panchenko, M.V. (2013). Histone acetyl transferase (HAT) HBO1 and JADE1 in epithelial cell regeneration. *The American journal of pathology* 182, 152-162.

Iizuka, M., and Stillman, B. (1999). Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. *The Journal of biological chemistry* 274, 23027-23034.

Iizuka, M., Takahashi, Y., Mizzen, C.A., Cook, R.G., Fujita, M., Allis, C.D., Frierson, H.F., Jr., Fukusato, T., and Smith, M.M. (2009). Histone acetyltransferase Hbo1: catalytic activity, cellular abundance, and links to primary cancers. *Gene* 436, 108-114.

Ito, S., Horikawa, S., Suzuki, T., Kawauchi, H., Tanaka, Y., Suzuki, T., and Suzuki, T. (2014). Human NAT10 is an ATP-dependent RNA acetyltransferase responsible for N4-acetylcytidine formation in 18 S ribosomal RNA (rRNA). *The Journal of biological chemistry* 289, 35724-35730.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. *Science* 293, 1074-1080.

Jones, D.T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. *J Mol Biol* 292, 195-202.

Kornberg, R.D. (1974). Chromatin structure: a repeating unit of histones and DNA. *Science* 184, 868-871.

Kornberg, R.D., and Lorch, Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* 98, 285-294.

Kueh, A.J., Dixon, M.P., Voss, A.K., and Thomas, T. (2011). HBO1 is required for H3K14 acetylation and normal transcriptional activity during embryonic development. *Molecular and cellular biology* 31, 845-860.

Lalonde, M.E., Avvakumov, N., Glass, K.C., Joncas, F.H., Saksouk, N., Holliday, M., Paquet, E., Yan, K., Tong, Q., Klein, B.J., *et al.* (2013). Exchange of associated factors directs a switch in HBO1 acetyltransferase histone tail specificity. *Genes & development* 27, 2009-2024.

Lalonde, M.E., Cheng, X., and Cote, J. (2014). Histone target selection within chromatin: an exemplary case of teamwork. *Genes & development* 28, 1029-1041.

Larrieu, D., Britton, S., Demir, M., Rodriguez, R., and Jackson, S.P. (2014). Chemical inhibition of NAT10 corrects defects of laminopathic cells. *Science* 344, 527-532.

Liu, H., Ling, Y., Gong, Y., Sun, Y., Hou, L., and Zhang, B. (2007). DNA damage induces N-acetyltransferase NAT10 gene expression through transcriptional activation. *Mol Cell Biochem* 300, 249-258.

Luger, K., Rechsteiner, T.J., and Richmond, T.J. (1999). Preparation of nucleosome core particle from recombinant histones. *Methods in enzymology* 304, 3-19.

Marmorstein, R., and Zhou, M.M. (2014). Writers and readers of histone acetylation: structure, mechanism, and inhibition. *Cold Spring Harb Perspect Biol* 6, a018762.

Miotto, B., and Struhl, K. (2008). HBO1 histone acetylase is a coactivator of the replication licensing factor Cdt1. *Genes & development* 22, 2633-2638.

Miotto, B., and Struhl, K. (2010). HBO1 histone acetylase activity is essential for DNA replication licensing and inhibited by Geminin. *Molecular cell* 37, 57-66.

Moss, S.F., Krivosheyev, V., de Souza, A., Chin, K., Gaetz, H.P., Chaudhary, N., Worman, H.J., and Holt, P.R. (1999). Decreased and aberrant nuclear lamin expression in gastrointestinal tract neoplasms. *Gut* 45, 723-729.

Peterson, C.L., and Laniel, M.A. (2004). Histones and histone modifications. *Curr Biol* 14, R546-551.

Piche, B., and Li, G. (2010). Inhibitor of growth tumor suppressors in cancer progression. *Cell Mol Life Sci* 67, 1987-1999.

Piperno, G., LeDizet, M., and Chang, X.J. (1987). Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. *J Cell Biol* 104, 289-302.

Ricketts, M.D., Frederick, B., Hoff, H., Tang, Y., Schultz, D.C., Singh Rai, T., Grazia Vizioli, M., Adams, P.D., and Marmorstein, R. (2015). Ubinuclein-1 confers histone H3.3-specific-binding by the HIRA histone chaperone complex. *Nature communications* 6, 7711.

Rojas, J.R., Trievel, R.C., Zhou, J., Mo, Y., Li, X., Berger, S.L., Allis, C.D., and Marmorstein, R. (1999). Structure of Tetrahymena GCN5 bound to coenzyme A and a histone H3 peptide. *Nature* 401, 93-98.

Saha, R.N., and Pahan, K. (2006). HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis. *Cell Death Differ* 13, 539-550.

Saksouk, N., Avvakumov, N., Champagne, K.S., Hung, T., Doyon, Y., Cayrou, C., Paquet, E., Ullah, M., Landry, A.J., Cote, V., *et al.* (2009). HBO1 HAT complexes target chromatin throughout gene coding regions via multiple PHD finger interactions with histone H3 tail. *Molecular cell* 33, 257-265.

Sapountzi, V., and Cote, J. (2011). MYST-family histone acetyltransferases: beyond chromatin. *Cell Mol Life Sci* 68, 1147-1156.

Shahbazian, M.D., and Grunstein, M. (2007). Functions of site-specific histone acetylation and deacetylation. *Annual review of biochemistry* 76, 75-100.

Sharma, S., Langhendries, J.L., Watzinger, P., Kotter, P., Entian, K.D., and Lafontaine, D.L. (2015). Yeast Kre33 and human NAT10 are conserved 18S rRNA cytosine acetyltransferases that modify tRNAs assisted by the adaptor Tan1/THUMP1. *Nucleic acids research* 43, 2242-2258.

Shen, Q., Zheng, X., McNutt, M.A., Guang, L., Sun, Y., Wang, J., Gong, Y., Hou, L., and Zhang, B. (2009). NAT10, a nucleolar protein, localizes to the midbody and regulates cytokinesis and acetylation of microtubules. *Exp Cell Res* 315, 1653-1667.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., *et al.* (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* 7, 539.

Smith, E.R., Eisen, A., Gu, W., Sattah, M., Pannuti, A., Zhou, J., Cook, R.G., Lucchesi, J.C., and Allis, C.D. (1998). ESA1 is a histone acetyltransferase that is essential for growth in yeast. *Proc Natl Acad Sci U S A* 95, 3561-3565.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. *Nature* 403, 41-45.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. *Genes & development* 12, 599-606.

Suzuki, T., and Miyata, N. (2006). Epigenetic control using natural products and synthetic molecules. *Curr Med Chem* 13, 935-958.

Tao, Y., Zhong, C., Zhu, J., Xu, S., and Ding, J. (2017). Structural and mechanistic insights into regulation of HBO1 histone acetyltransferase activity by BRPF2. *Nucleic acids research* 45, 5707-5719.

Taverna, S.D., Li, H., Ruthenburg, A.J., Allis, C.D., and Patel, D.J. (2007). How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Nat Struct Mol Biol* 14, 1025-1040.

Thomas, T., and Voss, A.K. (2007). The diverse biological roles of MYST histone acetyltransferase family proteins. *Cell cycle* 6, 696-704.

Turner, B.M. (2000). Histone acetylation and an epigenetic code. *Bioessays* 22, 836-845.

Verdin, E., and Ott, M. (2015). 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat Rev Mol Cell Biol* 16, 258-264.

Weissmann, F., Petzold, G., VanderLinden, R., Huis In 't Veld, P.J., Brown, N.G., Lampert, F., Westermann, S., Stark, H., Schulman, B.A., and Peters, J.M. (2016). biGBac enables rapid gene assembly for the expression of large multisubunit protein complexes. *Proc Natl Acad Sci U S A* 113, E2564-2569.

Worman, H.J., and Bonne, G. (2007). "Laminopathies": a wide spectrum of human diseases. *Exp Cell Res* 313, 2121-2133.

Wu, J., Wang, J., Li, M., Yang, Y., Wang, B., and Zheng, Y.G. (2011). Small molecule inhibitors of histone acetyltransferase Tip60. *Bioorg Chem* 39, 53-58.

Xu, P., Li, C., Chen, Z., Jiang, S., Fan, S., Wang, J., Dai, J., Zhu, P., and Chen, Z. (2016). The NuA4 Core Complex Acetylates Nucleosomal Histone H4 through a Double Recognition Mechanism. *Molecular cell* 63, 965-975.

Yan, Y., Barlev, N.A., Haley, R.H., Berger, S.L., and Marmorstein, R. (2000). Crystal structure of yeast Esa1 suggests a unified mechanism for catalysis and substrate binding by histone acetyltransferases. *Molecular cell* 6, 1195-1205.

Yan, Y., Harper, S., Speicher, D.W., and Marmorstein, R. (2002). The catalytic mechanism of the ESA1 histone acetyltransferase involves a self-acetylated intermediate. *Nature structural biology* 9, 862-869.

Ythier, D., Larrieu, D., Brambilla, C., Brambilla, E., and Pedoux, R. (2008). The new tumor suppressor genes ING: genomic structure and status in cancer. *International journal of cancer* 123, 1483-1490.

Zheng, Y.G., Wu, J., Chen, Z., and Goodman, M. (2008). Chemical regulation of epigenetic modifications: opportunities for new cancer therapy. *Med Res Rev* 28, 645-687.

Zhou, M.I., Wang, H., Ross, J.J., Kuzmin, I., Xu, C., and Cohen, H.T. (2002). The von Hippel-Lindau tumor suppressor stabilizes novel plant homeodomain protein Jade-1. *The Journal of biological chemistry* 277, 39887-39898.